Glycoconjugate Journal 17, 607–616, 2000

Hyaluronan, a common thread

Vincent C. Hascall

Department of Biomedical Engineering, ND-20, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195

Hyaluronan, nature's simplest, but still exceptionally versatile glycosaminoglycan, is currently the focus of attention across a wide front of research; from cell biology, morphogenesis, matrix organization, pathobiology to tissue engineering. This macromolecule has entangled me in a number of puzzling and challenging projects over the past 3 decades. These entertaining encounters are outlined in this retrospective.

Keywords: **hyaluronan, proteoglycan, aggrecan, link protein**

Introduction

Throughout my career, a common thread has woven itself into the fabric of my research, creating fascinating new patterns and dimensions. This is surprising because the thread, hyaluronan, has a rather simple, indeed common, structure that was defined by Karl Meyer's laboratory in an elegant series of experiments between its discovery in the vitreous humor of the eye [1] and the final definition of the repeating disaccharide, as described by Weissman and Meyer in 1954 [2].

-glucuronic acid-beta-1,3-N-acetylglucosamine-beta-1,4-

The following retrospective describes my personal encounters with this fascinating macromolecule.

Encounter 1 – The stealth macromolecule (1968)

Stanley Sajdera, a fellow graduate student, and I shared a basement laboratory at the Rockefeller Institute (now University) along with a cohort of very large cockroaches. In the mid 1960s, under the mentorship of Dr. Dominic Dziewiatkowski, we initiated a series of experiments to isolate a proteolytic activity from bovine nasal cartilage. My experiments investigated the chemical and physical properties of a proteinpolysaccharide (an original name for proteoglycan) that had been isolated from high-speed homogenates of the same tissue and that was being used as a substrate for Stan's enzyme activity. Stan was exploring the effects of reducing agents and denaturants, including 4 M guanidine HCl, on the isolation and activity of the enzyme. In a key experiment, he included a control extraction of cartilage slices with 4 M guanidine HCl alone. To our complete surprise, almost all of the proteoglycans were released into the extracting solution, even though the slices remained intact. This result led to the development of the dissociative extraction protocols now commonly used to solubilize proteoglycans [3].

A subsequent series of experiments using equilibrium density gradient fractionations and analytical centrifugal analyses demonstrated that the extraction mechanism involved dissociating proteoglycan aggregates in the cartilage, thereby allowing the proteoglycan monomer (now called aggrecan) to diffuse into the extraction solvent. Dialysis of the guanidine HCl to lower, associative concentrations allowed the aggregates to re-form. It was also clear that a glycoprotein, referred to as the glycoprotein link protein (now known as the link protein), was necessary and, so we thought at the time, sufficient for aggregation [4]. We had no idea, however, that the extracts also contained small amounts of a stealth macromolecule, hyaluronan. Therefore, we proposed the model for aggregation shown in Figure 1. In this model, the glycosaminoglycan chains were distributed along a central portion of the core protein of the proteoglycan with globular domains at either end – an unwitting precursor for the current model for the proteoglycan versican. Our experiments indicated that there were two distinct binding interactions involved in aggregation, and although both dissociated in 4 M guanidine HCl, only one was sensitive to low pH and reducing agents. Thus, the two globular domains in the model differed and interacted with one of two distinct sites in the link protein, thereby creating an alternating bead-like structure. Of course, without the stealth macromolecule, the model was entirely wrong, though we considered it pretty clever at the time.

Encounter 2 – The missing link (1973)

I met Dick Heinegard in 1972 at a Gordon Conference organized by Al Dorfman and Karl Meyer. After the conference, Dick visited my laboratory at the University of Michigan

To whom all correspondence should be addressed. Vincent C. Hascall. Tel.: +216-445-5676; E-mail: hascall@bme.ri.ccf.org

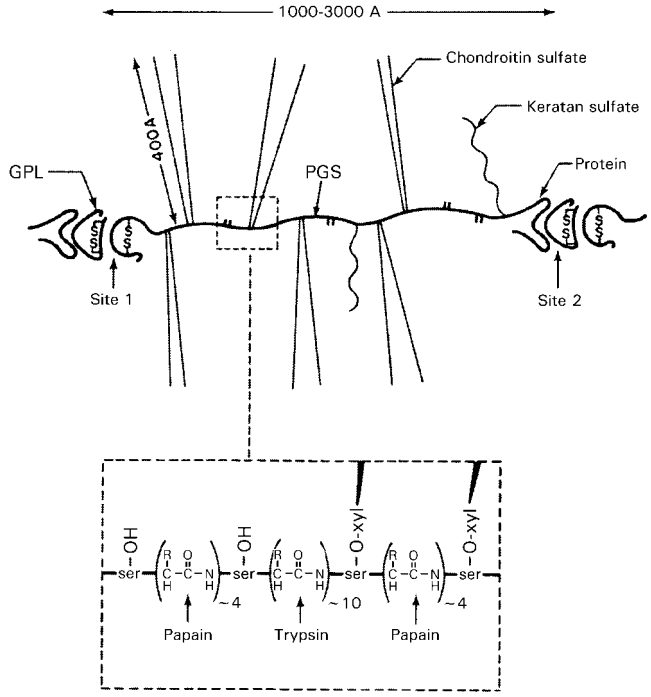

Figure 1. The model, taken from my thesis, proposed that aggregation of the proteoglycan subunit (PGS, alias aggrecan) was mediated by a glycoprotein link (GPL, alias link protein). Experimental evidence indicated two interactions. The site 1 interaction was reversed by reduction of disulfide bonds, chaotropic agents and acid pH. The site 2 interaction was reversed by chaotropic agents, but was stable to reduction of disulfide bonds and acid pH. The arrangement of chondroitin sulfate chains in 'doublets' along the core protein reflects the prevailing model at that time based upon Martin Mathew's work with trypsin and papain digests of proteoglycans [37]. This model was subsequently corrected in a study with Dick Heinegard [38]. The dimensions of the proteoglycan were based upon my measurements of transport properties of purified PGS (sedimentation velocity centrifugation and viscosity), and are surprisingly accurate all things considered.

and asked if I would like to spend a year in his laboratory at the University of Lund, Sweden. When I did so a year later, we had just learned of the important discovery by Hardingham and Muir [5] that the addition of small amounts of hyaluronan to a solution of proteoglycan monomer in associative solvents led to a rapid and dramatic increase in viscosity, indicating the formation of aggregates. However, the aggregates did not appear to be stable under conditions of ultracentrifugation that Stan and I had shown appropriate for analysis of re-formed native aggregates. Thus, Dick and I undertook two lines of investigation to unravel this puzzle. He introduced me to a very large molecular sieve column, through which we eluted partial digests of hyaluronan with testicular hyaluronidase to purify hyaluronan oligosaccharides of different sizes. These were then used to demonstrate that decasaccharides, but not octasaccharides, were able to decrease the viscosity of mixtures of proteoglycan monomer with hyaluronan, but were unable to decrease the viscosity of re-formed native aggregates [6]. The second line of experiments demonstrated

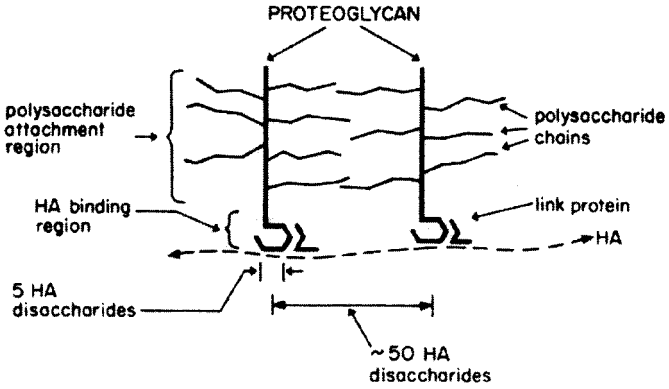

Figure 2. The insinuation of hyaluronan into the aggregate led to a correct structure with three distinct interactions: a globular portion of aggrecan (the G1 domain) interacting with hyaluronan, an adjacent link protein interacting with hyaluronan, and portions of each protein interacting with each other. In the context of the model in Figure 1, the interaction between the two proteins remains stable to reduction of disulfide bonds and acid pH. (From Reference 7 with permission of the publisher.)

that digestion of native aggregates with proteases, notably trypsin [7], left two proteins bound to the hyaluronan. One was the link protein, and the other was derived from a domain of the core protein of aggrecan, now referred to as the G1 domain.

These results led to the correct ternary model for the aggregates (Figure 2). Both the link protein and the G1 domain were proposed to have two binding interactions: one in each for interacting with hyaluronan with specificity for a stretch of hyaluronan 10 sugars long, and one in each for interacting with each other.

Encounter 3 – Get it all together now (1978)

Tim Hardingham came to my laboratory at NIH for a year and worked with Jim Kimura on a series of experiments to determine how chondrocytes organize aggregates from newly synthesized components. This study utilized defined sizes of hyaluronan oligosaccharides to probe the mechanism by which newly synthesized and secreted aggrecan molecules assemble into link protein-stabilized aggregates. As expected, decasaccharides were able to slow, but not prevent aggregate formation, whereas oligosaccharides of length ~28 or longer prevented aggregate formation [8] (Figure 3). These observations showed clearly that aggrecan and link protein only encountered hyaluronan after their secretion from the cell, and that the hyaluronan-binding sites in an adjacent link protein and G1 domain in an aggregate are within about 8 sugars along the hyaluronan chain. Jim also argued, and finally convinced us, that the data also provided good evidence that the link protein and the G1 domain can associate with each other before interacting with hyaluronan, possibly even before they leave the chondrocyte. Definitive proof for this has yet to be demonstrated, however. The mechanism by which the

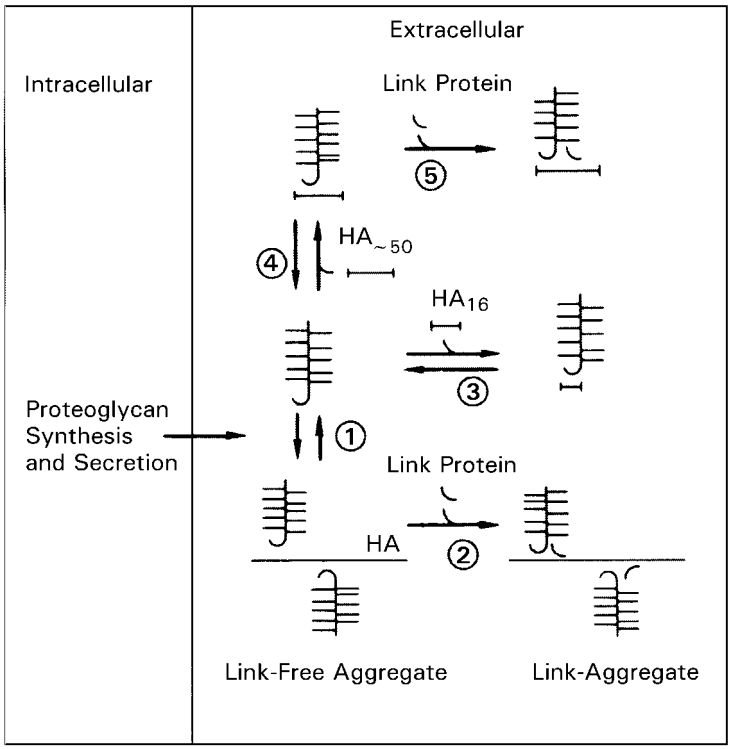

Figure 3. In this model, aggrecan and link protein are secreted from the chondrocyte separately from hyaluronan. In pathway 1 and 2, aggrecan interacts reversibly with hyaluronan and then is stabilized by addition of the link protein. In the presence of small hyaluronan oligosaccharides (10- to ~26-mers), aggrecan interacts reversibly with the oligomer (pathway 3). This inhibits, but does not prevent aggrecan molecules from forming stable aggregates by returning through pathway 1 and 2. In the presence of larger hyaluronan oligosaccharides, the oligomer can also accommodate addition of a link protein (pathway 4 and 5). This forms a stable 'aggregate of one' and prevents re-entry into pathway 1 and 2. (From Reference 8 with permission of the publisher.)

hyaluronan was synthesized and segregated from aggrecan and the link protein until outside the cell remained a puzzle.

Encounter 4 – No hook to hang on (1981)

It is still not straightforward to study the synthesis of hyaluronan. Radiolabeled glucosamine is commonly used, but the metabolic conversion of glucose to glucosamine introduces a specific activity dilution of intracellular glucosamine that is very difficult to measure or monitor [9]. For example, chondrocyte cultures can rapidly deplete glucose from the medium over a 24-h period, during which the specific activity of the intracellular glucosamine pool can vary greatly. Roger Mason initiated our attempts to study the biosynthesis of hyaluronan during his sabbatical at NIH. He used [3H]glucosamine as a precursor in chondrosarcoma chondrocyte cultures for labeling both the chondroitin sulfate on aggrecan and hyaluronan [10]. We assumed that the UDP-N-acetylhexosamine pool was a common precursor for the synthesis of both glycosaminoglycans and that the ratio of tritium in each was a direct measure of their relative masses synthesized during the labeling period, independent of any changes in the specific activity of the pool. Although this has been a useful method in many subsequent studies, this assumption may need to be revisited if the hyaluronan synthase is insulated from the cytosol as indicated in Encounter 9 below.

We also assumed that hyaluronan would be synthesized as a proteoglycan with a defined core protein, and Roger initiated a series of careful and technically difficult experiments with the chondrocytes using [3H]leucine as a precursor to identify it. As usual with assumptions about hyaluronan, this one proved to be wrong. Our best efforts failed to identify any covalently attached protein on newly synthesized hyaluronan molecules [11]. For this reason, hyaluronan does not fit the definition of a proteoglycan, but nevertheless is often referred to as an 'honorary' proteoglycan anyway. This result indicated that the mechanism of biosynthesis of hyaluronan must be uniquely different from that for the other classes of glycosaminoglycans.

Encounter 5 – Vanishing act (1985)

Late in 1981, I returned to NIH from a sabbatical year at Monash University in Australia. While there, I had worked with Chris Handley to develop a bovine cartilage explant model that maintains steady state metabolism of proteoglycans over several weeks. The half-life of newly synthesized aggrecan, labeled with radiosulfate, was shown to be about 20 days in the explant cultures [12]. Shortly after my return, Teresa Morales joined my laboratory and decided to use the culture

model to study the technically much more challenging question of how hyaluronan is catabolized in the explants. We felt that a likely outcome would be that the half-life of hyaluronan in the matrix would be much longer than that of the proteoglycans, which might come and go while the hyaluronan backbone of the aggregates remained in place in the matrix. Once again, this hypothesis proved to be wrong.

The experimental design required analysis of native aggregates. Therefore, it was essential to avoid the usual dissociation-reaggregation methods for isolating aggregates. To accomplish this, Teresa used associative solvents to extract native aggregates from thin frozen sections, and centrifugation in equilibrium density gradients and on sedimentation velocity gradients to purify them, recovering ~60% of the total originally present in the tissue [13]. The first surprise was that native aggregates isolated from cultures at different times after an initial labeling pulse with [3H]glucosamine and [35S]sulfate lost tritium in hyaluronan at the same rate as for the loss of tritium and radiosulfate in aggrecan with a half-life of ~20 days. This result indicated that the catabolism of both aggrecan and hyaluronan in native aggregates involves a coordinate mechanism. The second surprise was that no labeled hyaluronan was recovered in the medium from the cultures, although ~95% of the chondroitin sulfate of aggrecan was. The hyaluronan simply vanished during the catabolic process.

In 1992 and 1993, analyses of the catabolized aggrecan fragments in three laboratories identified the precise cleavage site in the aggrecan core protein that led to their release from the tissue [14,15,16]. These results, in combination with those of Teresa, led to the 'slurp' model of catabolism shown in (Figure 4) [17] (originally proposed in 1993 in a chapter for a book that was never published!). The model requires a catabolic unit at or near the chondrocyte cell surface with at least three coordinated activities: 1) a hyaluronan-binding protein (now identified as CD44 [18]), 2) a protease (aggrecanase) to cleave the aggrecan (now identified as a member of the ADAMTS family [19]), and 3) a hyaluronidase (yet to be identified) to break the chain and initiate internalization (slurping) of the attached portion of the hyaluronan for eventual complete degradation in the lysosome. The released fragment of aggrecan diffuses from the tissue into the medium or, *in vivo*, into the synovial fluid.

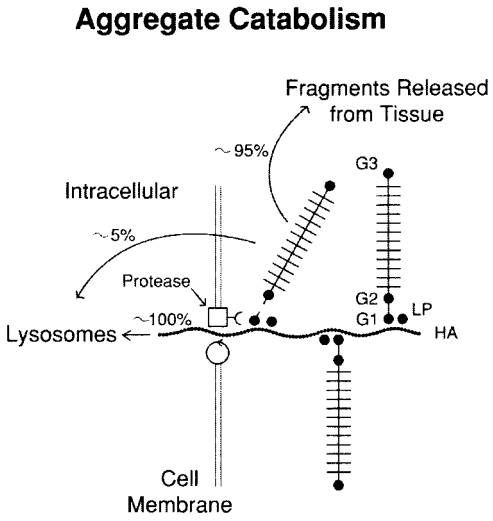

Figure 4. This unpublished 1993 model proposed that a catabolic unit present on or in the chondrocyte plasma membrane is responsible for aggrecan catabolism. The circle represents a hyaluronan-binding complex with hyaluronidase activity that cuts and internalizes the hyaluronan chain. The square represents a protease (aggrecanase) that cleaves a particular site between the G1 and G2 domains of aggrecan. Most of the unbound fragment with the glycosaminoglycan chains then diffuses out of the tissue. In explant cultures only 5% of these fragments are internalized, whereas all of the hyaluronan is. A modernized version of the model is presented in Reference 17.

Encounter 6 – Expanding horizons (1988)

Our research with hyaluronan to this point had been pretty myopic, focusing entirely on chondrocyte models. The arrival of Antonietta Salustri into the laboratory changed that and expanded our horizons to new biologies and new dimensions. Antonia's interest was in the process of cumulus oophorus expansion in the pre-ovulatory follicle. She had followed Masaki Yanagishita's work on proteoglycans synthesized by ovarian granulosa cells and, with him, initiated a course of studies on cumulus cell-oocyte complexes that continue to the present. For Antonia, a culture meant placing 20 mouse complexes (~20 000 cumulus cells) in 50 microliters of medium under polysiloxane and encouraging them to expand ~20 fold over a 20-h period of incubation (Figure 5).

Hyaluronan was known to be a key component of the expanded matrix, and Antonia adapted the dual-label method [9] to her microscale cultures to quantitate hyaluronan during the expansion process. Synthesis of hyaluronan is first observed between 2–3 h after initiating expansion, increases to a plateau level by ~6 h, begins to decrease at 10–12 h, and becomes

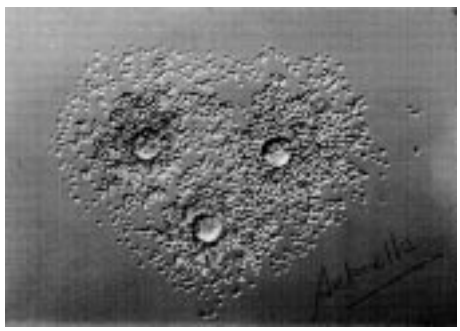

Figure 5. Antonella Camaioni came to NIH from Antonia Salustri's laboratory to work on cumulus oophorus expansion for a couple of years. In 1993, she gave this figure to me as a Valentine Day card. It shows three highly expanded cumulus cell-oocyte complexes that had been cultured for 18 h *in vitro*, under conditions promoting hyaluronan synthesis and matrix formation. Her work demonstrated that hyaluronan decasaccharides, but not octasaccharides prevent formation of the matrix [39].

undetectable by 16–18 h [20]. The net accumulation is ~4 ng per complex [21]. Three factors were required: 1) either follicle stimulating hormone (FSH) or epidermal growth factor (EGF), 2) a soluble factor produced by the oocyte [22], and 3) a serum factor identified by William Larsen's laboratory [23] as inter-alpha-trypsin inhibitor. The first two factors were shown to be required to up-regulate hyaluronan synthesis; the third is necessary for retaining the hyaluronan in the matrix surrounding the cumulus cells. FSH (or EGF) exerts all of its effects in the first 2 h after addition to a culture, a time that precedes the onset of hyaluronan synthesis, whereas the oocyte factor is required continuously to sustain hyaluronan synthesis until it ceases [24]. Clearly, regulation of hyaluronan synthesis in this critical biological process is complicated, involving several intracellular signaling pathways.

Encounter 7 – Ups and downs (1991)

Steve Evanko, a graduate student in Kate Vogel's laboratory at the University of New Mexico, came to NIH for a summer internship to work with Ron Midura and to learn methods for analyzing proteoglycans. Ron was investigating the properties of an osteoblastic cell line that synthesized bone sialoprotein, a heavily glycosylated and sulfated molecule suspected of being involved in matrix mineralization. The effects of parathyroid hormone on bone dynamics are complex with an interplay between bone forming and bone removing cells. The cell line provided a unique opportunity to determine how a uniform population of bone forming cells responds to the hormone. Therefore, Steve initiated a project to study its effects on the synthesis of bone sialoprotein and proteoglycans. However, the results with [35S]sulfate as a monitor unexpectedly showed only a slight increase in synthesis of bone sialoprotein and proteoglycans in the presence of the hormone over a 24-h period. Nevertheless, the net incorporation of tritium with [3H]glucosamine as a precursor was significantly increased, but not into either of these macromolecules. A few column analyses revealed that the increased tritium was almost entirely in hyaluronan. This led to the key experiment shown in Figure 6 in which cultures were labeled in discrete windows at times after adding parathyroid hormone [25]. Hyaluronan synthesis went up rapidly over the first 5 h and then down to the basal level between 10 and 15 h, a pattern reminiscent of cumulus cell-oocyte complex expansion.

Encounter 8 – Inside-outside Tango (1995)

This complex regulation of hyaluronan synthesis, first turning it on and then shutting it off, is emerging as a common theme in the response of cells to a variety of stimuli and poses numerous interesting questions. A key to answering them, however, was to identify the enzyme(s) responsible for its synthesis. This was the problem that Csaba Fulop chose when

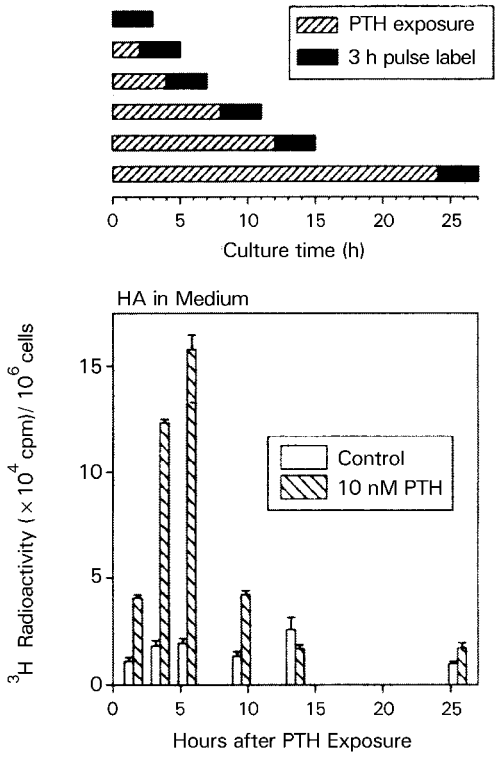

Figure 6. The ~6-fold increase of hyaluronan synthesis at 5 h after stimulating osteoblastic cells with a physiological level of parathyroid hormone and the subsequent rapid decline to basal level is characteristic of many cellular responses to a variety of stimuli. Transcriptional regulation of a hyaluronan synthase is the likely underlying mechanism. (From Reference 25 with permission of the publisher.)

he joined my laboratory at the Cleveland Clinic, where I had moved in 1994.

Earlier work by Peter Prehm had provided evidence that hyaluronan synthesis occurs at the plasma membrane, with the growing chain being extruded into the extracellular environment [26]. Further, his data suggested that elongation of the chain occurred at the reducing end by adding the alternate UDP-sugar substrate while displacing a UDP moiety from the terminus (Figure 7). This mechanism contrasts with that for elongating the other glycosaminoglycans, which occurs at their non-reducing ends, and it clearly obviates the necessity for a core protein. Still, the actual enzyme(s) remained elusive in spite of the efforts in several laboratories.

The lock was opened with the key identification of the bacterial hyaluronan synthase (HAS) [27]. Its homology to developmentally-regulated gene 42 (DG42), a protein with unknown function previously identified in Xenopus [28], provided an opportunity for Csaba to search for the eukaryotic enzyme through the use of degenerate primers based upon conserved regions of the sequences and RT-PCR [29]. Unknown to us, Andrew Spicer in John McDonald's laboratory used the same strategy and identified mouse hyaluronan synthase 2 (HAS2) [30] just before we did. Two other HAS

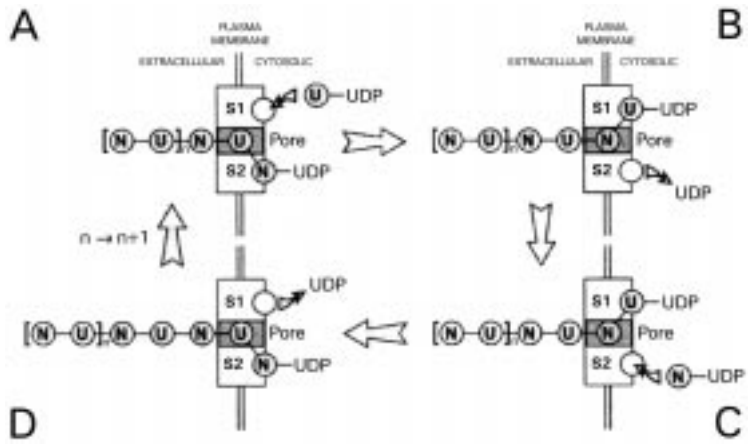

Figure 7. The proposed mechanism for hyaluronan synthesis is a complex sequence of events involving two sites for addition of the alternating sugars, translocation of the acceptor chain with UDP on the reducing terminus from one site to the other after addition of the respective alternate sugar from the UDP-sugar donors, and translocation of the growing chain through a pore into the extracellular space. One cycle as demonstrated from A to D increases the length of the chain by one disaccharide unit. This is a lot for a single protein to accomplish! (From Reference 40 with permission from the publisher.)

enzymes were also identified in the same time period. See Reference 31 for a review of the discovery of the eukaryotic HAS family.

Csaba used RT-PCR to show that has2 mRNA is: not detectable in cumulus cell-oocyte complexes at the initiation of an ovulatory cycle; barely detectable at 1 h; prominent at 3–5 h when hyaluronan synthesis is high; and back near basal levels by 13 h after the complexes have ovulated (Figure 8). More recent work with quantitative RT-PCR demonstrated that the maximum copy number of has2 mRNAs is only ~250 per cumulus cell at 4 h. These results show that transcriptional regulation of HAS2 controls hyaluronan synthesis in this model. They also indicate that very few has2 mRNAs, and hence probably very few HAS2 enzyme molecules, are necessary to sustain a high level of hyaluronan synthesis. Further, they suggest that mechanisms exist for turning over HAS2 enzymes within short time frames.

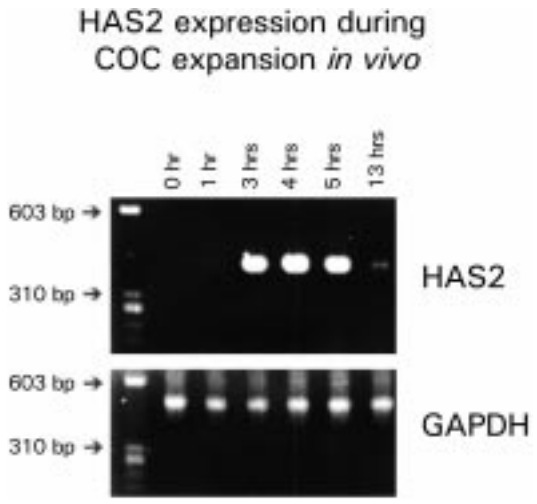

Figure 8. Cumulus cell-oocyte complexes were isolated at the indicated times after initiating an ovulatory cycle by injecting human chorionic gonadotropin into mice primed 48 h previously with pregnant mares' gonadotropin. Primers for has2 mRNA were used in RT-PCR analyses of RNA isolated from the complexes. (Unpublished work by Csaba Fulop.)

Encounter 9 – The hole story (1996)

'The experiment didn't work!' Nicola Goodstone was rather chagrined, but it was just an indication that hyaluronan was about to challenge our preconceptions yet again. Nikki joined the laboratory a year or so after my move to Cleveland and chose to work with Tony Calabro on a problem that I considered very risky. She proposed to use a bacterial toxin, alpha-hemolysin, to create small pores in the plasma membrane of the chondrosarcoma chondrocytes that would allow small molecules, including ATP and nucleotide-sugars, to diffuse out of the cells. In the absence of ATP and nucleotide-sugar substrates, the presumption was that hyaluronan synthesis would come to a halt, but that it could be reactivated *in situ* by adding exogenous substrates in sufficient quantities into the medium. The labeling precursors used were the standard [3H]glucosamine and [35S]sulfate.

'Well, what happened?' ATP flowed out of the cells, and then back in as it was used up, reaching levels in the cultures below the detection limit of the assay by ~8 h [32]. Incorporation of [35S]sulfate into chondroitin sulfate rapidly decreased during the same time period. However, tritium incorporation into hyaluronan continued at nearly the same level as in control cultures even at 24 h (Figure 9). The experiment had worked beautifully; it was just that the hyaluronan synthesis machinery, unlike that for chondroitin sulfate synthesis, was indifferent to depletion of cytosolic ATP and nucleotide-

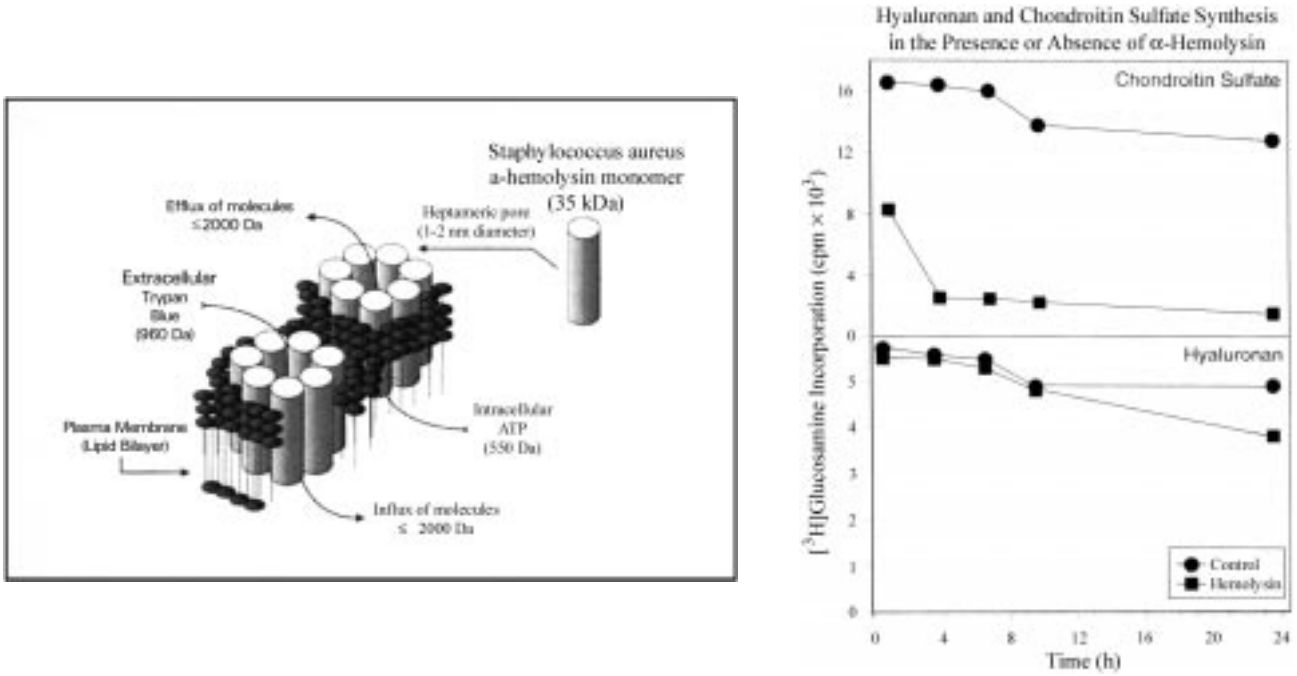

Figure 9. Alpha-hemolysin forms heptameric pores in the plasma membrane that allow small molecules to enter and leave the cytoplasm as indicated by the model on the left. The graphs on the right show the amounts of label from the [3H]glucosamine precursor recovered in chondroitin sulfate (upper) and hyaluronan (lower) during 2 h labeling windows in chondrocyte cultures at different times after initiating the experiment. As expected, synthesis of chondroitin sulfate in permeabilized cells declines rapidly. Remarkably, synthesis of hyaluronan is sustained at high levels even 24 h later. (Model on left from Reference 40 with permission of the publisher; Graph on right from Ref. [32] with permission of the publisher.)

sugars! However, the hyaluronan synthesis machinery is not indifferent to a source of metabolic energy, as was also demonstrated by showing that the synthesis of both hyaluronan and chondroitin sulfate showed parallel dose-dependent decreases when the cells were exposed to the ATP poison arsenate.

The results suggest rather strongly that there is a functional compartmentalization of a hyaluronan synthesis complex separate from the cytosol that must contain a source of energy and enzymes to convert glucosamine and glucose to the HAS2 substrates, UDP-N-acetylglucosamine and UDP-glucuronic acid, respectively. Proof of this concept will no doubt require several more experiments that 'don't work'.

Encounter 10 – To space or not to space (1997)

When I think of hyaluronan (which I do frequently, as anyone who has read this far might guess), I often think of volume (Figure 10). The physical properties of a polyanionic and very large polymer in solution quite naturally assume a domain structure that extends through a large volume of solvent. Small molecules move readily through such a solution, but larger molecules are greatly impeded by the domain structures. This is almost intuitive to someone who teethed as a graduate student on measuring transport properties of large macromolecules such as aggrecan. Thus, I was confronted with a dilemma. Before they joined my laboratory for a sabbatical, Markku and Raija Tammi had shown that, surprisingly (yet again), hyaluronan was present at considerable concentrations in the human epidermis, ~90 microgram per gram wet weight [33]. The problem was where to put it. Textbook histology of the epidermis shows that keratinocytes have virtually no

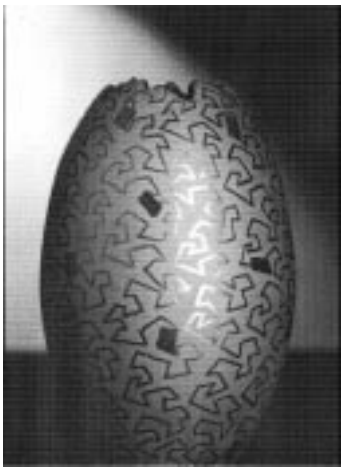

Figure 10. The vase occupies a 3-dimensional space, and it is easy to imagine that the artist was thinking about hyaluronan while designing the motif, which undoubtedly represents a partially expanded random coil hyaluronan molecule sprinkled with square proteins.

extracellular space around them, with measurements reflecting less than 1% of the total tissue volume outside the cell. If this amount of hyaluronan were distributed in less than 1% of the volume of the tissue, its concentration would be very high, nearly 10 mg/ml. This value is considerably more than other tissues, including the vitreous humor of the eye, ~3 mg/ml. This suggests that the extracellular space around keratinocytes in the epidermis is, in fact, more than the conventional histology would indicate.

That this may well be the case has been demonstrated in electron micrographs of human epidermis taken by Ernst Hunziker [34] (Figure 11). The tissue used for the upper micrograph was fixed and processed by conventional procedures, and that for the lower was from tissue processed by a freeze-substitution method designed to retain the relationship of cells and their surrounding matrix. It is clear that the latter procedure reveals a much more extensive extracellular space (~14%) than is observed with the conventional procedure (~2%). Most likely, conventional procedures extract much of the hyaluronan during the processing steps, allowing the extracellular space to collapse.

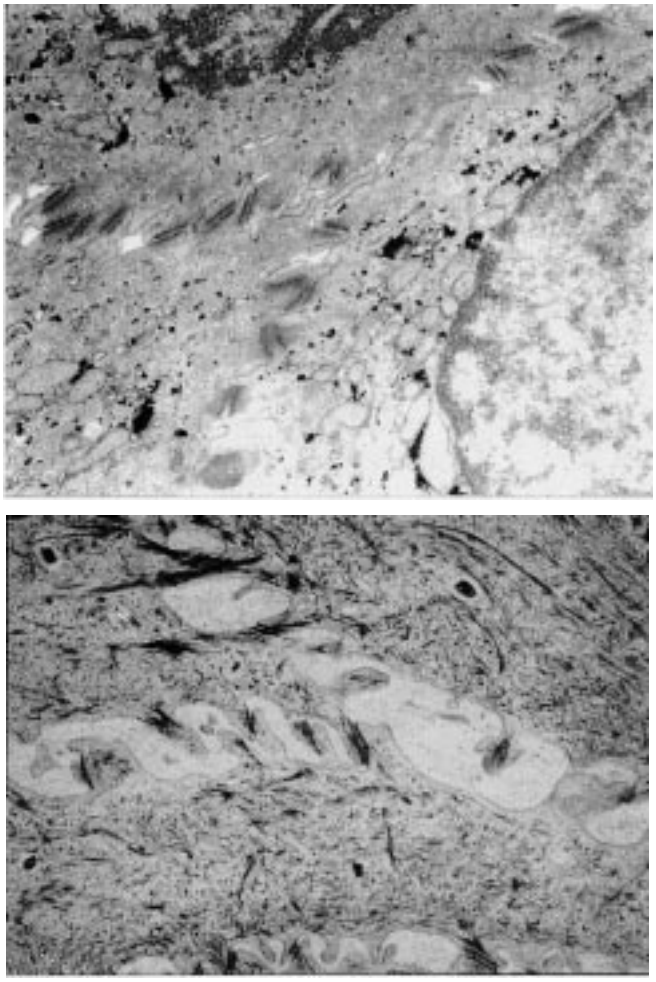

Figure 11. Human breast skin was either processed by conventional fixation, dehydration and embedding methods (upper), or by a freeze substitution method designed to retain the relationship of cells and their surrounding matrix (lower). The proportion of extracellular space around the keratinocytes is much greater when the tissue is processed by the freeze substitution method. (Electron micrographs kindly provided by Ernst Hunziker.)

In collaboration with Don MacCallum at the University of Michigan, Markku and Raija used a rat epidermal keratinocyte cell line to explore the metabolism and function of hyaluronan in a culture model of an epidermis. When these cells are cultured 'lifted' at an air-medium interface on an open collagen substrata, or on a collagen substrata with a superimposed basement membrane, they differentiate, stratify, undergo apopotosis and form a stratum corneum. In short, they form an epidermis that is histologically indistinguishable from a thin section of a murine epidermis [35].

When the collagen is open, most of the newly synthesized hyaluronan escapes into the substrata, whereas in the presence of a superimposed basement membrane, it is mostly retained in the cell layer. In the latter case, experiments show that [3H]glucosamine equilibrates with the hyaluronan pool in the cell layer within 24 h, indicating that the half-life of a newly synthesized hyaluronan molecule is indeed short in this tissue. A similar half-life for labeled hyaluronan was also observed with explant cultures of skin [33]. Thus, metabolism of hyaluronan in the epidermis is remarkably active with biosynthetic and catabolic mechanisms balanced to maintain a steady state concentration around the cells.

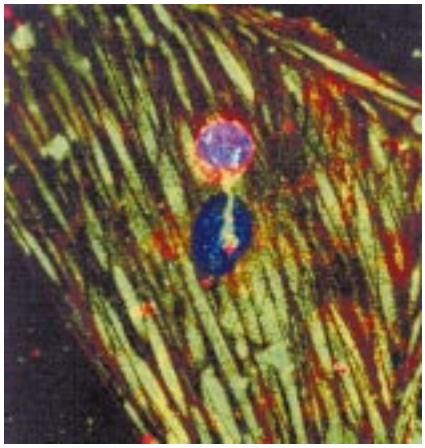

Figure 12. This figure provides some lovely color for the last encounter described in this article, but it is also a paradigm underlying a dreadful disease, Crohn's inflammatory bowel disease. The mononuclear leukocyte has engaged the green hyaluronan cable projecting above the surface of the underlying smooth muscle cell from human colon tissue. The red CD44 molecules on the surface are involved in binding the leukocyte to the hyaluronan. Interestingly, the leukocytes seldom interact with the green patches of hyaluronan that coat the surface of the smooth muscle cell, which confronts us with a current hyaluronan conundrum. (See also the cover of the June, 2000 issue of Molecular Biology of the Cell.) (Confocal micrograph kindly provided by Carol de la Motte, Scott Strong and Judy Drazba.)

Encounter 11 – Balloon on a string (1999)

A visitor suggested that the confocal micrograph in Figure 12 should be titled 'Balloon on a String' while another suggested 'Around the World in 80 Days'. On the other hand, it illustrates a puzzle underlying our most recent encounter with hyaluronan. It also reflects a novel mechanism involved in inflammatory bowel disease. The green patches of hyaluronan are anchored on the surface of the smooth muscle cell from human colon primarily through binding to the cell surface molecule, CD44, which contains a hyaluronan-binding motif homologous to those in the link protein. The thick green cable rising from the cell surface above the nucleus is another hyaluronan structure with different organizational properties. The monocyte has attached to the end of the cable structure by a mechanism involving CD44 (stained red) on its surface, but has not bound to the hyaluronan in the patches. What is different about these two hyaluronan configurations?

This mystery began when Carol de la Motte, an investigator in Scott Strong's laboratory in Colorectal Surgery at the Cleveland Clinic stopped by my office to discuss some puzzling results. Their project involved treating cultures of normal smooth muscle cells from human colon tissue with inflammatory cytokines or with viruses (or more routinely with the viral analogue, double-stranded poly I:C RNA) and monitoring their ability several hours later to bind mononuclear leukocytes. The response to transforming necrosis factor-alpha (TNF-alpha) was well worked out; VCAM-1 was up-regulated on the surface of the smooth muscle cell and was recognized by its binding partner, the VLA-4 integrin, on the monocytes. A blocking antiserum to VCAM-1 prevented monocyte binding. The mystery was with the response of the smooth muscle cells to virus infection or to poly I:C treatment. VCAM-1 was up-regulated and considerably more monocytes bound, yet the blocking antiserum to VCAM-1 had little effect in preventing binding. As I knew very little about any of the players in this drama, I was at a loss as to what to recommend.

Carol then indicated that there is an intriguing difference. The monocytes that bind after cytokine stimulation adhere closely to the smooth muscle cell surfaces, whereas those that bind after poly I:C treatment appear loosely adherent and tethered like beads on a string. 'Try hyaluronidase,' was my response, and the enzyme indeed released most of the monocytes. Subsequent experiments showed that monocytes pretreated with a blocking antibody to CD44 no longer bound to poly I:C treated smooth muscle cells, indicating that this enigmatic cell-surface molecule has a central role in the binding mechanism [36].

What is the relevance of this mechanism? One can imagine that a cell infected with a virus and in extremis turns to the indomitable hyaluronan synthetic factory to spin a final web with information to capture transient monocytes responsible for surveillance. Once alerted, the bound monocytes can activate and begin the process of assembling the appropriate array of defenders necessary to combat the invader. Perhaps this is a rather normal response mechanism that unfortunately is not properly shut down in patients with Crohn's disease.

Close encounter of the twelfth kind – 2001 – A space Odyssey

Our goal as scientists is to discover the future. In my case I hope to discover the future of yet more hyaluronan puzzles, and perhaps to solve some that remain on the shelf. I do not expect to be disappointed in this quest. After all, this common thread has never been shy about dashing my preconceptions.

Stay tuned and take a look now and then at the 'Science of Hyaluronan Today' in the website (glycoforum.gr.jp) to keep abreast with the latest developments of this elusive macromolecule.

Acknowledgment

I am most grateful to Ms. Christine Kassuba for her excellent editorial suggestions during the course of preparing this article.

References

1 Meyer K, Palmer JW, The polysaccharide of the vitreous humor, *J Biol Chem* **107**, 629–34 (1934).
2 Weissman B, Meyer K, The structure of hyalobiuronic acid from umbilical cord, *J Am Chem* **76**, 1753–57 (1954).
3 Sajdera SW, Hascall VC, Proteinpolysaccharide complex from bovine nasal cartilage: A comparison of low and high shear extraction procedures, *J Biol Chem* **244**, 77–87 (1969).
4 Hascall VC, Sajdera SW, Proteinpolysaccharide complex from bovine nasal cartilage: The function of glycoprotein in the formation of aggregates, *J Biol Chem* **244**, 2384–96 (1969).
5 Hardingham TE, Muir H, The specific interaction of hyaluronic acid with cartilage proteoglycans, *Biochim Biophys Acta* **279**, 401–5 (1972).
6 Hascall VC, Heinegard D, Aggregation of cartilage proteoglycans. II. Oligosaccharide competitors of the proteoglycan-hyaluronic acid interaction, *J Biol Chem* **249**, 4242–9 (1974).
7 Heinegard D, Hascall VC, Aggregation of cartilage proteoglycans. III. Characteristics of the proteins isolated from trypsin digests of aggregates, *J Biol Chem* **249**, 4250–6 (1974).
8 Kimura JH, Hardingham TE, Hascall VC, Solursh M, Biosynthesis of proteoglycans and their assembly into aggregates in cultures of chondrocytes from the Swarm rat chondrosarcoma, *J Biol Chem* **254**, 2600–9 (1979).
9 Yanagishita M, Salustri A, Hascall VC, Specific activity of radiolabeled hexosamines in metabolic labeling experiments, *Methods in Enzymology* **179**, 435–45 (1989).
10 Mason RM, Kimura JH, Hascall VC, Biosynthesis of hyaluronic acid in cultures of chondrocytes from the Swarm rat chondrosarcoma, *J Biol Chem* **257**, 2236–45 (1982).
11 Mason RM, d'Arville C, Kimura JH, Hascall VC, Absence of covalently linked core protein from newly synthesized hyaluronate, *Biochem J* **207**, 445–57 (1982).

12 Hascall VC, Handley CJ, McQuillan DJ, Hascall GK, Robinson HC, Lowther DA, The effect of serum on biosynthesis of proteoglycans by bovine articular cartilage in culture, *Arch Biochem Biophys* **224**, 206–23 (1983).
13 Morales TI, Hascall VC, Correlated metabolism of proteoglycans and hyaluronic acid in bovine cartilage organ cultures, *J Biol Chem* **263**, 3632–8 (1988).
14 Ilic MZ, Handley CJ, Robinson HC, Mok MT, Mechanism of catabolism of aggrecan by articular cartilage, *Arch Biochem Biophys* **294**, 115–22 (1992).
15 Sandy JD, Neame PJ, Boynton RE, Flannery CR, Catabolism of aggrecan in cartilage explants: Identification of a major cleavage site within the interglobular domain, *J Biol Chem* **266**, 8683–5 (1991).
16 Loulakis P, Shrikhande A, Davis G, Maniglia CA, N-terminal sequence of proteoglycan fragments isolated from medium of interleukin-1-treated articular cartilage cultures: Putative site(s) of enzymatic cleavage, *Biochem J* **284**, 589–93 (1992).
17 Hascall VC, Sandy JD, Handley CJ, Regulation of proteoglycan metabolism in articular cartilage. In *The Biology of the Synovial Joint*, edited by Archer CW, Caterson B, Benjamin M, Ralphs JR (Harwood Academic Publishers, Netherlands, 1999), pp. 110–20.
18 Chow G, Knudson CB, Homandberg G, Knudson W, Increased expression of CD44 in bovine articular chondrocytes by catabolic cellular mediators, *J Biol Chem* **270**, 27734–41 (1995).
19 Tortorella MD, Burn TC, Pratta MA, Abbaszade I, Hollis JM, Liu R, Rosenfeld SA, Copeland RA, Decicco CP, Wynn R, Rockwell A, Yang F, Duke JL, Solomon K, George H, Bruckner R, Nagase H, Itoh Y, Ellis DM, Ross H, Wiswall BH, Murphy K, Hillman MC Jr, Hollis GF, Amer EC et al. Purification and cloning of aggrecanase-l: a member of the ADAMTS family of proteins, *Science* **284**, 1664–6 (1999).
20 Salustri A, Yanagishita M, Hascall VC, Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification, *J Biol Chem* **264**, 13840–7 (1989).
21 Salustri A, Yanagishita M, Underhill CB, Laurent TC, Hascall VC, Localization and synthesis of hyaluronic acid in the cumulus cells and mural granulosa cells of the preovulatory follicle, *Dev Biol* **151**, 541–51 (1992).
22 Salustri A, Ulisse S, Yanagishita M, Hascall VC, Hyaluronic acid synthesis by mural granulosa cells and cumulus cells in vitro is selectively stimulated by a factor produced by oocytes and by transforming growth factor-beta, *J Biol Chem* **265**, 19517–23 (1990).
23 Chen L, Mao SJ, Larsen WJ, Identification of a factor in fetal bovine serum that stabilizes the cumulus extracellular matrix. A role for a member of the inter-alpha-trypsin inhibitor family, *J Biol Chem* **267**, 12380–6 (1992).
24 Tirone E, D'Alessandris C, Hascall VC, Siracusa G, Salustri A, Hyaluronan synthesis by mouse cumulus cells is regulated by interactions between follicle-stimulating hormone (or epidermal growth factor) and a soluble oocyte factor (or transforming growth factor-betal), *J Biol Chem* **272**, 4787–94 (1997).
25 Midura RJ, Evanko SP, Hascall VC, Parathyroid hormone stimulates hyaluronan synthesis in an osteoblast-like cell line, *J Biol Chem* **269**, 13200–6 (1994).
26 Prehm P, Hyaluronate is synthesized at plasma membranes, *Biochem J* **220**, 597–600 (1984).
27 De Angelis PL, Papaconstantinou J, Weigel PH, Molecular cloning, identification, and sequence of the hylauronan synthase gene from group *A Streptococcus pyogenes*, *J Biol Chem* **268**, 19181–4 (1993).
28 Rosa F, Sargent TD, Rebbert ML, Michaels GS, Jamrich M, Grunz H, Jonas E, Winkles JA, Dawid IB, Accumulation and decay of DG42 gene products follow a gradient pattern during Xenopus embryogenesis, *Dev Biol* **129**, 114–23 (1988).
29 Fulop C, Salustri A, Hascall VC, Coding sequence of a hyaluronan synthase homologue expressed during expansion of the mouse cumulus-oocyte complex, *Arch Biochem Biophys* **337**, 261–6 (1997).
30 Spicer AP, Augustine ML, McDonald JA, Molecular cloning and characterization of a putative mouse hyaluronan synthase, *J Biol Chem* **271**, 23400–6 (1996).
31 Weigel PH, Hascall VC, Tammi M, Hyaluronan synthases, *J Biol Chem* **272**, 13997–14000 (1997).
32 Goodstone NJ, Hascall VC, Calabro A, Differential effects of *Staphylococccus aureus* alpha-hemolysin on the synthesis of hyaluronan and chondroitin sulfate by rat chondrosarcoma chondrocytes, *Arch Biochem Biophys* **350**, 26–35 (1998).
33 Tammi R, Saamanen A-M, Maibach HI, Tammi M, Degradation of newly synthesized high molecular mass hyaluronan in the epidermal and dermal compartments of human skin in organ culture, *J Invest Derm* **97**, 126–30 (1991).
34 Hascall VC, Tammi R, Tammi M, Hunziker E, MacCallum DK, Does keratinocyte hyaluronan determine the volume of extracellular space in the epidermis? In *New Frontiers in Medical Sciences*: *Redefining hyaluronan*, edited by Abatangelo G, Weigel PH (Elsevier Science, 2000), pp. 31–40.
35 Tammi RH, Tammi MI, Hascall VC, Hogg M, Pasonen S, MacCallum DK, A pre-formed basal lamina alters the metabolism and distribution of hyaluronan in epidermal keratinocyte 'organotypic' cultures grown on collagen matrices, *Histochem Cell Biol* **113**, 265–77 (2000).
36 De la Motte CA, Hascall VC, Calabro A, Yen-Lieberman B, Strong SA, Mononuclear leukocytes preferentially bind via CD44 to hyaluronan on human intestinal mucosal smooth muscle cells after virus infection or treatment with poly (I:C), *J Biol Chem* **274**, 30747–55 (1999)
37 Mathews MB, Comparative biochemistry of chondroitin sulphate-proteins of cartilage and notochord, *Biochem J* **125**, 37–46 (1971).
38 Heinegard D, Hascall VC, Characterization of chondroitin sulfate isolated from trypsin-chymotrypsin digests of cartilage proteoglycans, *Arch Biochem Biophys* **165**, 427–41 (1974).
39 Camaioni A, Hascall VC, Yanagishita M, Salustri A, Effects of exogenous hyaluronic acid and serum on matrix organization and stability in the mouse cumulus cell-oocyte complex, *J Biol Chem* **268**, 20473–81 (1993).
40 Calabro A, Midura RJ, Hascall VC, Plaas A, Goodstone NJ, Roden L, Structure and biosynthesis of chondroitin sulfate and hyaluronan. In *Proteoglycans – structure, biology and molecular interactions*, edited by Iozzo RV (Marcel Dekker, 2000), pp. 5–26.