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Hyaluronan in adipogenesis, adipose tissue physiology and systemic metabolism

Yi Zhu[a], **Ilja L. Kruglikov**[b], **Yucel Akgul**[c], and **Philipp E. Scherer**[a,d]

[a]–Touchstone Diabetes Center, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[b]–Wellcomet GmbH, Karlsruhe, Germany

[c]–Department of Plastic Surgery, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[d]–Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

Abstract

Hyaluronic acid (HA, also known as hyaluronan), is a non-sulfated linear glycosaminoglycan polymer consisting of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine abundantly present in the extracellular matrix. The sizes of hyaluronic acid polymers range from 5000 to 20,000,000 Da *in vivo*, and the functions of HA are largely dictated by its size. Due to its high biocompatibility, HA has been commonly used as soft tissue filler as well as a major component of biomaterial scaffolds in tissue engineering. Several studies have implicated that HA may promote differentiation of adipose tissue derived stem cells *in vitro* or *in vivo* when used as a supporting scaffold. However, whether HA actually promotes adipogenesis *in vivo* and the subsequent metabolic effects of this process are unclear. This review summarizes some recent publications in the field and discusses the possible directions and approaches for future studies, focusing on the role of HA in the adipose tissue.

Keywords

Hyaluronan; Hyaluronic acid; Adipose tissue; Adipogenesis; Extracellular matrix; Dermal filler

Introduction

Hyaluronan (HA) is a non-sulfated linear glycosaminoglycan polymer consisting of repeating disaccharide units of β−1,4 linked D-glucuronic acid (GlcUA) and β−1,3 linked *N*-acetyl-D-glucosamine (GlcNAc). HA is secreted to the extracellular matrix in most of mammalian tissues. It is synthesized by three plasma membrane-bound hyaluronan synthases, HAS1, HAS2 and HAS3. During their synthesis, the nascent HA chains are

Correspondence to Philipp E. Scherer: Touchstone Diabetes Center, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. Philipp.Scherer@UTSouthwestern.edu.

extruded through pore-like structures into the extracellular space [1]. Newly synthesized HA can be processed by hyaluronidases (HYALs) or broken down non-enzymatically by reactive oxygen species [2]. Hyaluronidases hydrolyze the hexosaminidic β(1–4) linkage between GlcNAc and GlcUA of the HA chain and release small HA fragments.

The half-life of HA differs in different organs [3,4]. The turnover of HA is extremely high in circulation. In humans, the plasma half-life of HA is estimated to be about 2–6 min, resulting in a total turnover of 10–100 mg per day [5], The whole body HA turnover in various tissues is estimated to take place within 3 days with about 5 g per day turned over [6]. HA synthesis and degradation is also very dynamic at the cellular level. In cells, normal HA synthesis is activated transiently for cell division or motility, after which HA is rapidly cleared from the site by endocytic uptake and hyaluronidase-catalyzed hydrolysis [7].

Cosmetic use of hyaluronan

HA has high cross-species structural homology, which makes HA synthesized in bacteria or other species non-antigenic and non-immunogenic in humans [8]. This property enables its widespread application for cosmetic uses [9]. In fact, HA plays a central role in the dermal filler industry. By itself, it is the agent of choice for wrinkle fillers, preferred over collagens or other categories of smaller synthetic wrinkle fillers [10]. HA fillers are also useful in repairing scars or in other conditions, such as HIV-associated lipodystrophies [11,12] and steroid atrophy [13]. Most HA-fillers are derived from bacterial culture to ensure very low protein contamination, and multiple cross-linking approaches have been developed to increase its stability. Unwanted side effect of HA fillers are rare, and the effects last from several months to over a year [10,14].

HA fillers are predominantly injected into the subcutaneous adipose tissue, and deeper injections lead to prolonged efficacy [15]. Longevity of the volumizing effect after injection of HA fillers demonstrates high inter-subject variations, no correlation was found between the longevity of volumizing effect and the tissue hyaluronidase level [16]. Skin improvements can be observed even after full biodegradation of the filler. Moreover, the longevity of HA filler effects may be connected with some long-term structural modification of the adipose tissue [17].

Physical properties of hyaluronan solutions that can influence the state of the adipose tissue

HA has a very high affinity for water molecules. At the same time, it is a soluble polymer which is normally rapidly resorbed after injection. Its stability and mechanical properties are improved if HA is cross-linked or bound to collagens when used as a cosmetic dermal filler.

The concentration of HA in different tissues varies significantly, being up to 500 μg/g in human skin, up to 5 mg/g in uterine cervix at late pregnancy, up to 3 mg/mL in synovial fluid, and up to 100 ng/mL in blood serum [18,19]. In hypertrophic adipose tissue, HA was measured in concentration up to 16 pg/cell [20], which for a cell with a diameter of about 100 μm corresponds to a volume concentration of about 30 μg/g. Since HA in adipose tissue

is mainly concentrated in the pericellular space around the adipocyte, local concentrations of the HA around hypertrophic adipocytes are likely to be much higher.

The relative concentration of HA is of primary importance for the biophysical properties of an HA solution, since HA molecules behave as highly hydrated random coils, which start to entangle at concentrations of approximately 1 mg/mL [21]. Above the entanglement point, the viscosity of HA solution rapidly increases with increasing HA concentration c (exponentially, as c3.3), and the HA solution becomes gel-like. This behavior can significantly influence the proliferative and differentiative properties of adipose tissue stem cells (ASCs) [22]. The elasticity of the HA gel also increases with increasing molecular weight and concentration of HA.

The osmotic pressure in an HA gel is dependent on its concentration and ionic strength of the solution, J, as $\Pi = Ac^{9/4}J^{-3/4}$, where A is about 1.4×10^3 kPa, and c and J are expressed in mole [23]. This behavior corresponds to earlier reported experimental results that the HA solutions with concentrations of 5 mg/mL, 10 mg/mL, and 20 mg/mL have the osmotic pressures of about 1 mm Hg, 4.5 mm Hg and 18 mm Hg, respectively [24]. This osmotic pressure can be further increased if HA is connected with collagen, which is the case in WAT, where the pericellular HA is connected with Col VI. Since the concentration of HA can increase more than twice in WAT of diet-induced and genetically obese mice compared to controls [20], this phenomenon can lead to an approximately 4.75-fold increase of the osmotic pressure in the gel. Such an increase of osmotic pressure can strongly decrease the transcapillary transport in WAT, thus creating conditions similar to those observed in solid tumors [25]. The osmotic pressure in an HA gel can be reduced if the solution contains salts with high ionic strength [23]. For example, in a solution containing 100 mM NaCl + 100 mM $CaCl_2$, the osmotic pressure in an HA gel will be reduced 3.5-fold compared to a solution containing 200 mM NaCl. A recent report indicated that differentiating preadipocytes (which are known to produce high levels of HA during differentiation) demonstrate reduced fat deposition in the presence of higher concentrations of NaCl [26].

Once the cell experiences an increase in the external osmotic pressure, water flows out of the cell, its volume and turgor decreases and the cell shrinks until a new osmotic equilibrium is reached. This can inflict significant damage on cells. To counter this damage, different types of cells rapidly produce and accumulate polyols, which, in the case of adipocytes, are present as glycerol. Glycerol accumulated intracellularly would leak out of the cell if glycerol transports *via* aquaglyceroporin channels, unless these channels are effectively inactivated. If this process persists, adipocyte would need to continuously synthesize glycerol or undergo lipolysis. This continuous glycerol efflux from adipocytes would enter the circulation and end up in the liver and other organs. This could be the underlying mechanism for body contouring. For example, additional adipose tissue HA is produced at high temperature during the body contouring treatment. The HA binds to a large amount of water that temporally improves the local skin texture while at the same time promotes the adipose tissue lipolysis; afterward, excessively accumulated water and exported glycerol and lipids will be cleared over the time, which leads to a circumference reduction effect.

Hyaluronan in adipogenesis

White adipocyte tissue grows by cellular hyperplasia and volume expansion during development and a calorie surplus. However, the origin of white adipocytes and developmental process, especially in adulthood, are complicated and remain to be completely understood, though much progress has been made in the recent past [27]. The prevailing hypothesis is that a perivascular population of cells which are high in Pdgfrβ and Zfp423 and resemble mural cells (pericytes and vascular smooth muscle cells) give rise to new adipocytes under pro-adipogenic conditions, such as high-fat diet treatment [28]. During maturation of preadipocytes, they progressively change their shape and accumulate lipid droplets, a process that needs to be coordinated with the remodeling of the extracellular matrix (ECM) to accommodate the expanding cellular volume and intercellular space [29]. *Vice versa*, adipogenesis is spatially and temporally regulated by ECM.

Changes in HA levels have been observed during differentiation of 3T3–L1 preadipocytes *in vitro* [30], hyperglycemia can even divert dividing osteoblastic precursor cells to a metabolically stressed adipogenic program, while at the same time inducing the synthesis of hyaluronan [34]. Supplementation of HA in culture medium prolonged lifespan, reduced cellular senescence, and enhanced differentiation potential of murine adipose tissue stromal cells [31]. In contrast, adipogenesis in 3T3–L1 cells was inhibited by reducing HA levels *via* treating them with exogenous hyaluronidase, or by inhibiting HA synthesis *via* 4-methylumbelliferone treatment or by reducing HAS2 levels [32]. It is important to note that most adipocytes are cultured under high-glucose and high-insulin conditions to promote differentiation and maintain their adipocyte identity [30], an environment that can promote inflammation and the generation of reactive oxygen species (ROS) [33]. These *in vitro* conditions, therefore, may not reflect physiological adipose tissue differentiation *in vivo*. Nevertheless, in a high-fat diet induced obesity mouse model, *in vivo* administration of exogenous hyaluronidase enzymes reduced abdominal fat accumulation and inhibited lipid accumulation in liver and thereby increased insulin sensitivity [32,35], implicating a possible role of HA in adipogenesis *in vivo*.

HA exerts many different biological functions on adipose tissue *via* binding to different cell surface proteins, including receptors such as CD44, RHAMM/HMMR, Brevican, TNFIP6, LYVE1 and SHAP [36–38]. CD44 is one of major cell surface binding proteins for HA [39], and the PDGFRα + CD44+ subpopulation of preadipocyte is highly proliferative [40]. Activation of RHAMM/HMMR receptor antagonizes the CD44 signaling and suppresses adipogenesis [41]. The adipogenic potential of HA combined with its physical properties makes it the top choice of supporting matrices for *in vivo* transplantation of preadipocytes or adipocyte stem cells (ASCs) [42–46]. A study that evaluated different scaffolds for human ASC allografts showed that differentiation of hASCs was augmented when the cells were encapsulated in cross-linked hyaluronan scaffolds gels [47]. A similar experiment performed in pigs showed the emergence of islets of mature adipocytes and neovascularization of the fat tissue arising from injected preadipocytes mixed with HA gels; interestingly, the degree of crosslinking by carboxyl groups amidation seems to be an important factor in determining the adipogenic potential of the HA gel [46]. HA scaffolds were also shown to decrease the necrosis of adipocytes during allografting in a rabbit model [48].

HA is also used in *in vivo* differentiation of "beige" or "brite" adipocyte, the third kind of adipocytes that are similar to brown adipocytes, rich in mitochondria and uncoupling protein-1 (UCP1) [49,50]. Beige adipocytes can emerge through transdifferentiation from white adipocytes controlled by sympathetic neuronal signals [51–53], or through *de novo* beige adipogenesis, also mediated by sympathetic input [54,55]. Expansion of UCP1 positive beige adipocytes that uncouple mitochondrial respiration from ATP synthesis has repeatedly been shown to be physiologically beneficial by reducing circulating glucose and lipid levels. However, in human adults, brown or beige adipose tissues are scarce, and cold-induction remains the most efficient method to induce beige adipose tissue thus far. It is therefore important to engineer large quantities of UCP1-positive beige adipocytes *in vivo*. Recent developments in formulating hyaluronic acid-based scaffolds have enabled functional tissue allografts [56], that support the *in vivo* differentiation of transplanted ASCs to beige adipose tissue with successful vascularization in the host [57].

Despite many positive results *in vitro* or in animal models, HA's pro-adipogenic effects have been questioned in some studies [58]. The challenge that these aforementioned studies face is to go back to the exact injection site to dissect the transplant tissue out for histological analysis. Many times the histological pictures are inconclusive in distinguishing adipocytes from other types of cells in the matrix, especially among cells loaded with lipid droplets and *bona fide* adipocytes.

One approach to more carefully dissect the role of HA in adipogenesis *in vivo* is utilizing the AdipoChaser mouse model we previously developed (Fig. 1A) [54]. After supplementing doxycycline in the diet for 4–5 days, all existing adipocytes are labeled in blue (after reacting with the substrate X-gal), then doxycycline is withdrawn, any new adipocytes emerging from that point forward remain unlabeled. To test the effects of the HA-based dermal filler Juvederm Ultra XC (Allergan, Santa Barbara) on adipogenesis *in vivo*, we used this mouse model and pre-labeled all adipocytes blue using doxycycline, and after complete doxycycline withdrawal, we injected Juvederm into the inguinal fat pad and switched mice to a high-fat diet to promote adipogenesis. 6 weeks later we dissected the fat pads injected with Juvederm and performed LacZ staining, and performed immunofluorescent staining for Perilipin1 (the slides were also counterstained with DAPI). We can clearly visualize the blue staining overlapping with the red Perilipin1 staining, indicating pre-existing, pre-labeled adipocytes. Within the Juvederm gel area, multiple nuclei are stained with DAPI, suggesting infiltration of cells into the Juvederm gel. However, none of those cells have positive Perilipin1 staining, suggesting none of them are mature adipocytes (Fig. 1B).

Clearly, this result should not be interpreted as evidence suggesting HA cannot promote adipogenesis *in vivo*. The Juvederm gel used in the study is a cross-linked HA with a very high HA concentration of 24 mg/mL, which should produce high osmotic pressure within the gel area and may pose a restrictive environment for adipose progenitor cells to expand and further develop into mature adipocytes. Future studies will have to test whether alternative HA mixtures are better, or test the effects in transgenic mouse models that overproduce HA locally. It is also important to point out that the inguinal fat pad is known to have very low adipocyte proliferation rates *in vivo*. There may be a very high barrier for adipogenesis to take place in this inguinal adipose depot. Pretreatment of mice with

tamoxifen, which stimulates *de novo* adipogenesis following transient lipoatrophy [59] may lower this barrier for HA to stimulate adipogenesis in the inguinal fat pad.

Furthermore, we cannot exclude that the cells infiltrating the HA gel are the immature adipocytes. Low molecular weight HA generated during degradation of HA gel is shown to induce chemokine expression in macrophages [60] and endothelial cells [61] through induction of CD44 and CXC1/GRO1 proteins. CXC1 is one of the dominant chemokines in preadipocytes [62], and it is recently shown that the immature adipocytes undergo chemotaxis through the activation of CXCL1 and CXCL8 [63]. From these observations, we can infer that the degradation of the HA gel and the production of low molecular weight fragments may induce the chemotaxis of immature adipocytes leading to their penetration into the HA gel.

HA effects on the proliferation of ASCs have also been demonstrated to be dose-dependent, with a maximal proliferation of these cells at an HA concentration corresponding to the HA entanglement point of about 1 mg/mL [22]. Further increase of HA concentrations above this point reduces the proliferation of ASCs which can be connected with rapidly increasing viscosity and osmotic pressure under such high HA concentrations. This can also explain some of the contradictory experimental results reported.

Hyaluronan in adipose tissue and systemic metabolism

Adipocyte hyperplasia and hypertrophy are hall-marks of obesity, which precedes the development of many metabolic diseases, including diabetes [64,65]. Accumulating evidence suggests a role of HA in this process. For example, the HA receptors RHAMM/HMMR and CD44 have been implicated in the development of diabetes. Especially, a genome-wide association study links the major HA receptor CD44 with type 2 diabetes [66]. Injection of an anti-CD44 monoclonal antibody makes mice more resistant to insulin-dependent diabetes mellitus [67], suggesting that the HA activated CD44 signaling is involved in the development of the disease. Beyond the mechanisms that we previously discussed as to how HA may alternate physical properties of adipose tissue and subsequently its metabolism, HA is also implicated in the modulation of extracellular matrix and inflammative states of adipose tissue and many other metabolic organs. Importantly, HA is one of the most reliable bio-markers for non-alcoholic fatty liver disease [68], with an especially strong predicting power in liver fibrosis when combined with other serum biomarkers such as procollagen III N-terminal peptide and TIMP1 [69]. Also, high levels of HA and inflammatory cells accumulate around diabetic pancreatic islets suggesting a role of HA in type 1 diabetes [70].

In diabetic patients, serum and tissue HA is elevated [71] as excess glucose in circulation and tissue enters hexosamine biosynthetic pathway to produce UDP-GlcNAc, an intermediate metabolite for HA synthesis [72]. The elevated intracellular UDP-GlcNAc levels also affect protein modification to affect cellular signaling such as PKC signaling [73] which leads to the increase of HAS2 expression [74,75], serving as a feed-forward mechanism to synergistically promote HA synthesis.

Treatment of HFD-induced insulin resistant mice with recombinant hyaluronidase PH20 (PEGPH20) reduced HA accumulation in muscle and improved whole-body insulin sensitivity [35]. Interestingly, treatment with PEGPH20 also resulted in up to 35% reduction in adipose tissue mass and a simultaneous reduction in adipocyte size [35]. The improvement of insulin sensitivity was attributed to better blood perfusion in skeletal muscle [35]. Whether the reduction in adipose tissue weight contributed to the improvement of insulin sensitivity is unknown, nor the direct effect of reducing HA in the adipose tissue.

Importantly, the metabolic outcome of HA is also a function of its size [38], and the lower molecular weight HA fragments have very different functions in contrast to the high molecular weight HA on adipose tissue. Small HA fragments produced by hyaluronidases can induce angiogenesis, an important component of adipose tissue healthy expansion. However, a recent study showed medium molecular weight (approx. 50 kDa) HA inhibits adipogenesis in cultured 3 T3–L1 cells [76], complicating the view on the role of different molecular weight HAs in adipogenesis. In a separate report from the same research group, the 50 kDa HA fragments have been shown to decrease adipogenic differentiation *in vitro* and *in vivo*. Oral administration of these HA fragments decreased body weight, adipose tissues, serum lipid (low-density lipoprotein cholesterol, triglyceride), and leptin levels in mice fed on a high-fat diet. HA fragments also decreased the hypertrophy of adipose tissue and ameliorated liver steatosis, showing a strong anti-obesity and anti-diabetic effect, possibly through enhancing PPARα and suppressing PPARγ expression [77]. It is important to note that the orally administrated HA may not enter circulation, so the site of action for the HA used in this study may be the intestinal digestive track.

These seemingly contradictory studies reveal the complexity of the function of HA. Previous studies largely rely on *in vitro* experiments. Even when *in vivo* experiments were performed, most of the time, it involved whole body genetic manipulations or drug treatments that affect simultaneously multiple organs in the research subjects. Tissue-specific and inducible systems will be more useful for a careful assessment of the functions of HA in various metabolic organs and the potential roles in multiple organ crosstalks. Doxycycline-inducible and tissue-specific HA synthase or hyaluronidase overexpression systems should serve this purpose and will be of great value to the studies of functions of HA in metabolic tissues, including the adipose tissue.

Concluding remarks

Decades of HA studies started from chemical structure, physical properties to the roles of HA in mediating immune response, with a good portion of the effects devoted to bioengineering of the HA as a scaffold for many biological applications. Recent advancements in genetics and the development of serum stable hyaluronidase enzymes have advanced our understanding of HA in the metabolism, especially the role of HA in adipogenesis and adipose tissue metabolism. Future studies should leverage the advancement in genetically engineered animal models that tissue specifically overexpress an HA synthase or a hyaluronidase to carefully dissect the roles of HA in the adipose tissue and other metabolic organs.

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The authors apologize for the inability, due to the enormous amount of literature on hyaluronan studies, to reference all studies relevant to this review.

Abbreviations used:

HA	hyaluronic acid
ECM	extracellular matrix
HYAL	hyaluronidase
ASCs	adipose tissue stem cells
UCP1	uncoupling protein-1
GlcUA	D-glucuronic acid
GlcNAc	*N*-acetyl-D-glucosamine
UDP	Uridine diphosphate.

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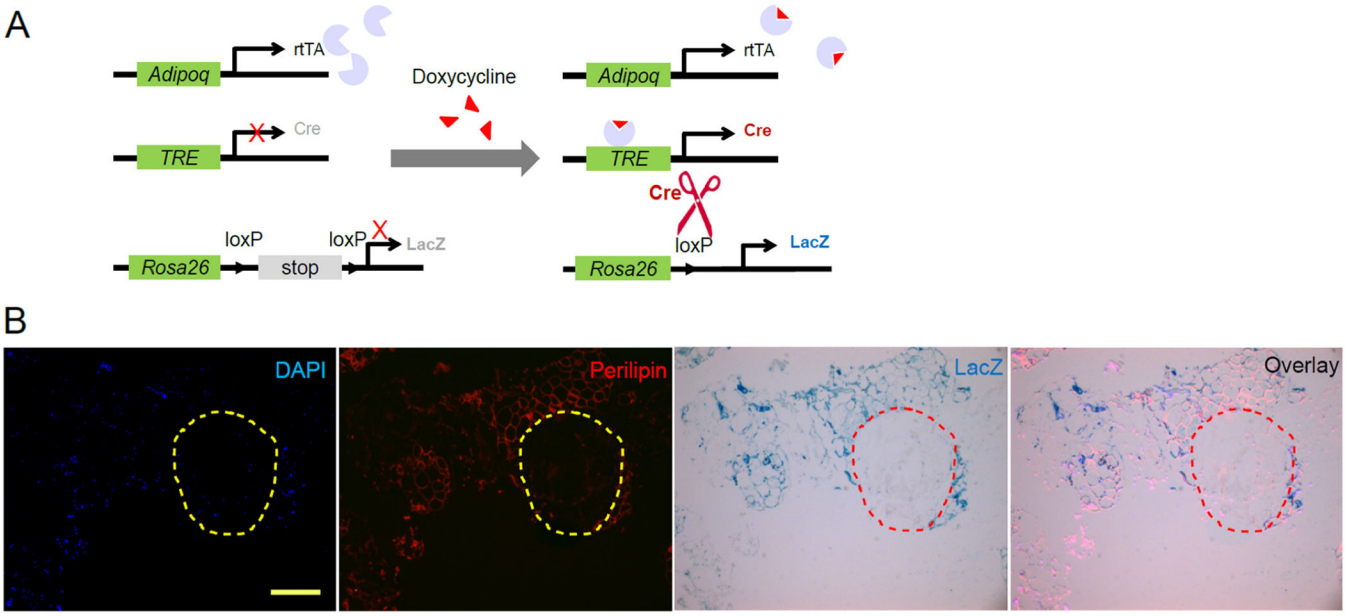

Fig. 1.

Detection of *de novo* adipogenesis using AdipoChaser mice. (A) Schematic graph of the AdipoChaser system. Adiponectin-rtTA (Adipoq-rtTA), TRE-Cre and Rosa26-loxP-stop-loxP-lacZ triple transgenic mouse is hereby called the AdipoChaser mouse. It constitutively expresses rtTA in mature adipocytes but only expresses Cre when doxycycline (dox) is supplemented. The Cre will subsequently recombine the loxP sites and remove the stop cassette to allow expression of LacZ. The LacZ expression will persist even after removal of dox. But new adipocytes emerging from non-adiponectin expressing progenitor or stem cells after doxycycline removal will not express LacZ. LacZ reacts with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and develops a dark blue color. So existing mature adipocytes will be labeled blue after dox supplementation, and any new adipocytes emerging after removal of dox will not be labeled. (B) Representative β-gal (blue) and Perilipin1 (red) staining of subcutaneous white adipose tissue in AdipoChaser mouse 6 weeks after Juvederm injection. Nuclei are counterstained with DAPI (blue). The circle indicates the boundary of Juvederm and adipose tissue. Scale bar: 250 μm.