Title: Evaluation of the in vivo kinetics and bio-stimulatory effects of subcutaneously injected hyaluronic acid filler

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Short running head
In vivo kinetics of injected HAF

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Nearly 20 years have passed since the first clinical trial report on hyaluronic acid filler (HAF).[1] HAFs have been mainly used in the field of cosmetic surgery for rejuvenation of the face and neck. In the treatment of facial wrinkles, HAF is usually injected into the skin using linear or sheet injection techniques such as linear threading, serial puncture, fanning, or cross-hatching.[2] Repeated treatments are often required every 6 months to a year because HAF is absorbed and metabolized.[3]

In the last decade, cosmetic treatment with HAF has succeeded in concealing facial depressions due to aging, in addition to augmenting the nose,[4] jaw, and cheeks.[5] HAFs have been further applied to the treatment of other conditions addressed in the field of plastic surgery, including facial soft-tissue degenerative diseases[6-8] and the reconstruction of recessed facial deformations following surgery or trauma.[9] In such treatments, HAF is injected as a bolus into the subcutaneous tissue or above the periosteum, providing a volume-augmenting effect that often persists for more than a year. One study reports that HAF injected into the face was found during surgical treatment 23 months later.[10] However, it remains unclear why the volume-augmenting effect can persist for such a long period after bolus injection. Several mechanisms may be involved, including the prevention of HAF absorption and metabolism via capsule formation and the maintenance of volume via autogenous tissue replacement. This study investigates the in vivo kinetics of bolus-injected HAF in rats. Understanding the changes that occur in HAF over time may provide information needed to develop an

effective injection method for maintaining long-term volume augmentation with HAF.

MATERIALS AND METHODS

Animals

Twenty-week-old female F344/NSlc inbred rats (Sankyo Labo Service Corp., Tokyo, Japan) were used in this study. This study received the approval of the ethics committee of Teikyo University Hospital for the performance of experiments on animals (Teikyo Medical Ethics, Issue 14-019, June 2014).

HAF

Juvederm Vista ULTRA PLUS (Allergan Japan KK, Tokyo, Japan) was used for this study. This product is a non-animal-derived hyaluronic acid produced by bacterial fermentation and chemical crosslinking.

Animal model

General anesthesia was induced in each rat by intraperitoneal injection of 30 mg/kg pentobarbital sodium (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan). In addition, local anesthesia was performed by cooling the back of the rat. A 28-gauge needle was inserted between the skin and the muscle layer, the needlepoint stopped, and 0.2 mL of HAF was subcutaneously injected as a bolus to obtain an elevating effect in local tissue. A macroscopic

image captured immediately after injection of HAF is seen in Fig. 1, *above*. Rats were euthanized by an excessive intraperitoneal injection of pentobarbital sodium (45 mg/kg) immediately after HAF injection (week 0) or at 4, 8, 16, 32, or 64 weeks later (n = 4 for each time point). The raised region on the back area, including the muscle layer into which the HAF had been injected, was immediately excised.

Magnetic resonance imaging (MRI) to examine the shape and volume of injected HAF

The specimens collected at each time point were examined using a permanent magnet type 1.5 T magnetic resonance imager (MR mini SA, DS Pharma Biomedical, Osaka, Japan) designed for small animals. The imaging sequence was performed using a T2-weighted 2D-MS method, with Repetition Time (TR)/Echo Time (TE) = 2000/69 ms and a slice thickness of 1.0 mm. The high intensity area corresponding to the HAF was measured using Image J (Ver. 1.47 V; freeware downloaded from http://www.updatestar.com/en/topic/Image%20j%201.47%20v). The area of each slice was multiplied by the slice thickness to obtain the volume, and the total HAF volume was calculated by adding the volumes of all cross sections. The height and width of the injected HAF was taken to be the maximum height and width found among all of the sections from each specimen. The average height and width was calculated for each time point.

Preparation of histological specimen sections

Tissue specimens containing HAF were fixed for over 24 h with 10% formaldehyde (Wako Pure Chemical Industries, Tokyo, Japan). Paraffin sections with a thickness of 4 μm were cut and mounted on slides. Hematoxylin-eosin (HE) stain and several other stains described below were applied. All sections were deparaffinized before staining, dehydrated after staining using 2-propanol (Kanto Chemical, Tokyo, Japan) and xylene (Muto Pure Chemicals, Tokyo, Japan), and sealed using malinol mounting medium (Muto Pure Chemicals).

Histological examination using Alcian Blue staining

Alcian blue staining was introduced to investigate the position and shape of the injected HAF in the rat back sample. Samples were immersed in 3% acetic acid solution (Wako Pure Chemical Industries) for 2 min and stained with Alcian Blue solution at pH 2.5 for 30 min. Samples were immersed again in 3% acetic acid solution and stained with nuclear fast red solution (Muto Pure Chemicals) for 5 min.

Histology for Elastica van Gieson (EVG) staining

EVG staining was performed to observe fibrotic changes in and around the injected HAF. Samples were immersed in resorcin-fuchsin solution (Muto

Pure Chemicals) for 15 min and stained with Weigert's iron hematoxylin stain solution for 6 min. Weigert's iron hematoxylin stain solution was mixed with equal amounts of preservative solution I, containing hematoxylin (Kanto Chemical) and 95% ethanol (Japan Alcohol, Tokyo, Japan), and preservative solution II, containing iron (III) chloride hexahydrate (Wako Pure Chemical Industries) and 23% hydrochloric acid (Muto Pure Chemicals). After processing with 0.5% hydrochloric acid alcohol (Wako Pure Chemical Industries), samples were stained with van Gieson's solution for 18 min. Van Gieson's solution was mixed with 100 mL picric acid-saturated solution (Wako Pure Chemical Industries) and 15 mL 1% acid fuchsin (Muto Pure Chemicals).

Immunohistochemical staining of type III collagen and Ki67

After heat treatment at 95 degrees Celsius and the application of an instant citrate buffer solution (LSI Medience, Tokyo, Japan) to activate sample antigens, samples were washed with phosphate-buffered saline (LSI Medience) and immersed in 3% hydrogen peroxide solution (Wako Pure Chemical Industries) for 5 min. Anti-collagen type III rabbit polyclonal antibody (Abcam) at a 1:400 dilution was used as the primary antibody and was allowed to react with the samples at room temperature for 50 min. Simple Stain Mouse MAX-PO (Nitirei Biosciences, Tokyo, Japan) was used as the secondary antibody at room temperature for 30 min. DAB (Super

Sensitive DAB; BioGenex Laboratories, Fremont, CA) was used for 5 min to develop the color. Finally, nuclear staining was performed for 1 min using Mayer's hematoxylin solution (Muto Pure Chemicals).

Nuclear Ki67 protein is a cellular proliferation marker[11] and was stained to examining fibroblast activity in the HAF. Anti-Ki67 polyclonal antibody (1:800) (Thermo Fisher Scientific, Inc. Waltham, MA) was used as the primary antibody, and one-step Polymer-HRP (BioGenex Laboratories) was used as the secondary antibody. DAB (Super Sensitive DAB, BioGenex Laboratories) was used to develop the color. Nuclear staining was performed using Mayer's hematoxylin solution (Muto Pure Chemicals).

Histological analysis of calcification using von Kossa staining

Von Kossa staining was conducted to examine the presence of calcification around the HAF, which may lead to capsule contracture. Samples were immersed in 5% silver nitrate (Wako Pure Chemical Industries) for 1 h and, stained with 5% sodium thiosulfate solution (Wako Pure Chemical Industries) for 3 min, and stained for 10 min in nuclear fast red solution.

Determination of the proportion of collagen fibers in the injected HAF space

Because collagen fibers are synthesized in the HAF, the proportion of collagen fiber and HAF-injected space in each sample were calculated at each time point. Since collagen fibers are stained reddish purple by EVG staining, the reddish purple area was extracted and measured using image processing software (BZ-X Analyzer BZ-H3C 1.3.0.3, KEYENCE, Osaka, Japan). The section with the largest area of HAF for each specimen was used for this measurement.

Statistical analysis

Data are expressed as the mean ± standard deviation. Using the statistical software SAS 9.4 (SAS Institute, Cary, NC), data from 0, 4, 8, 16, 32, and 64 weeks were analyzed between all possible pairs using Tukey's test. $P < 0.05$ (both sides) was considered a significant difference.

RESULTS

MRI findings

HAF was indicated as a high intensity area between the skin and the muscle layer. Hyaluronic acid exhibited a homogeneous area of high signal intensity immediately after injection, but the intensity gradually diminished after 4 weeks and was then imaged as a heterogeneous area (Fig. 2).

Immediately after injection, the volume of HAF was 195.8 ± 1.9 mm^3. With the mean value taken as 100%, the change in volume is shown in Fig. 3,

above. The HAF volume had increased to approximately 1.8 times the initial volume at 4 weeks after the injection. Although the mean volume consistently decreased thereafter, the volume at 16 to 64 weeks after injection did not differ significantly from the initial volume.

The maximum HAF height was 5.2 ± 0.4 mm, immediately after injection. With the mean value taken as 100%, changes in height are shown in Fig. 3, *center*. The greatest change occurred during the first 4 weeks: the HAF form became flattened, and the height was reduced by two-thirds.

The HAF width immediately after injection was 7.3 ± 1.0 mm. With this mean value taken as 100%, changes in the HAF width are shown in Fig. 3, *below*. Similar to the results for height, the greatest change occurred from immediately after injection to 4 weeks later, during which time the width increased 1.8-fold. Although the widths at 4 to 32 weeks after injection were significantly greater than the initial width, there was no significant difference between the initial width and that at 64 weeks.

Histological findings using Alcian blue and HE staining and immunohistochemical staining of Ki67

The image of Alcian blue staining at week 0 (Fig. 1, *below*) demonstrates that HAF was injected as a mass under the panniculus carnosus between the skin and muscle layers.

Histological examination using HE staining shows a proliferation of fibroblasts and adipocytes within the HAF at 4 weeks and later after

injection (Fig. 4, *above*). Immunohistochemical staining of Ki67 also indicates the proliferation of these cells in HAF (Fig. 4, *below*) (**See Figure, Supplemental Digital Content 1**). Fibroblasts and connective tissue created lattice structures within the HAF. During the late phase (32-64 weeks), HAF was further replaced by adipocyte-dominant autogenous tissues.

A capsule was present around the HAF at 4 weeks and subsequent time points. However, this capsule was not as thick as that seen around a silicon implant in vivo (**See figure, Supplemental Digital content 2**).

Histology using EVG staining and immunohistochemical staining for type III collagen

Temporal changes in EVG-stained collagen fibers and immunohistochemically-stained type III collagen are shown in Fig. 5. EVG stains collagen fibers reddish purple, and smooth muscles are stained yellow. Collagen fibers rapidly increased during the first 8 weeks. Luminal structures of the vascular smooth muscle were present after 4 weeks, indicating that angiogenesis occurred within the HAF (Fig. 5, *left column*). These structures were observed up to 64 weeks after injection. Immunostaining for type III collagen confirmed that these collagen fibers contained type III collagen (Fig. 5, *right column*), which appears during the initial process of wound healing.[12]

We also investigated changes in the proportion of collagen fibers within the HAF (Fig. 6). The proportion of collagen fibers was 1.2 ± 0.4%

immediately after injection and reached a peak of 21.5 ± 3.5% after 32 weeks. The presence of collagen fibers immediately after injection is thought to be due to contamination from the surrounding tissue at the time of injection.

Examination of calcifications in and around the HAF by von Kossa staining

We performed von Kossa staining to investigate whether calcification occurred in and around the injected HAF at each time point. No calcification was observed in any section (**See Figure, Supplemental Digital Content 3**).

DISCUSSION

The subcutaneous bolus injection of HAF was followed by an increase in its volume over the first 4-8 weeks caused by water absorption by the hydrophilic HAF. Simultaneously, lattice structures were created by fibrogenesis following the migration of fibroblasts and angiogenesis. In the late phase (32-64 weeks), adipocyte-dominant, autogenous tissue replacement within the HAF was observed. Although the HAF was gradually metabolized and absorbed, a portion of the HAF-injected space was replaced by autogenous tissue composed of fibroblasts, connective tissue, adipocytes, and vessels. These data are not directly applicable to human clinical cases because of differences between animal species with respect to skin tension, injection layers, and the type of HAF used. However, if patients treated with a subcutaneous bolus injection of HAF demonstrate long-lasting volume retention, these mechanisms might contribute to sustained volumizing

effects.

The MRI findings demonstrate that the HAF volume had increased to approximately 1.8 times the initial volume by 4 weeks after injection. Hyaluronic acid is hydrophilic; thus, the hyaluronan polymer absorbs the surrounding water, increasing its volume. Hyaluronic acid is a linear polymer of acidic mucopolysaccharide in which D-glucuronic acid and N-acetyl-D-glucosamine residues are alternately and repeatedly bonded.[13] Because hyaluronic acid has several hydroxyl groups, it expands as a coiled structure into solution. Water retention as much as 1000 times the initial mass of hyaluronic acid has been reported.[14, 15]

HE staining revealed that fibroblasts had migrated into the HAF by 4 weeks and lattice structures had formed (Fig. 4 and 5). EVG staining revealed that after 4 weeks, collagen fibers occupied 13.8% of the HAF area, on average.

After the maximum peak at 4 weeks, the average volume of hyaluronic acid gradually decreased. The shape of the HAF became markedly flatter during the first 4 weeks. The height decreased to approximately two-thirds of the initial height during the first 4 weeks, followed by a long period of without further change. Although the average HAF volume at 64 weeks was 74.8% of the initial volume, this difference is not statistically significant.

A previous study in animals reports that a fibrous capsule formed around hyaluronic acid subcutaneously injected as a bolus.[16] This feature may not be a problem as long as the capsule remains thin. However, one case

study reports that a large amount of hard-viscosity HAF injected for breast augmentation caused a capsule contracture.[17] At present, HAFs are available in different particle sizes, viscosities, and elasticities that result from different crosslinking and manufacturing processes.[18] Therefore, not all parameters were investigated in this study. However, neither a thick capsule, inflammatory reaction, nor calcification was noted around the HAF during the 64-week follow-up.

A previous study reports that 20-30% of hyaluronic acid is locally metabolized, with the remainder metabolized by the lymphatic pathway.[15] Although HAF is gradually metabolized, the lattice structures may allow the form to remain stable for a sufficient amount of time until the neogenesis of adipocytes, primarily observed during the late phase (at 32-64 weeks). MRI demonstrated that the homogeneous, high intensity signal exhibited by HAF at week 0 gradually became a heterogeneous signal in the late phase. This result also suggests that HAF was gradually replaced by autogenous tissues.

The migration and proliferation of fibroblasts into the HAF initiates the formation of lattice structures. Fibroblasts migrate into and proliferate in the HAF for several reasons, one of which is physical stimulation. The injection of HAF into the dermis generates mechanical stress from the extension of tissue, which is reported to activate fibroblasts and cause an increase in extracellular matrix constituents such as collagen, elastin, and proteoglycan in the dermis.[19] This change increases the activity of the TGF-ß pathway,[20-22] which is known to stimulate fibroblasts and endothelial cells,

inducing collagen, matrix formation, and blood vessels in soft-tissue wound-healing processes.[23] The subcutaneous injection of HAF may act as a trigger to activate fibroblasts, resulting in their migration into and proliferation in the HAF. In addition, fibroblasts are activated by hyaluronic acid itself.[24, 25] CD44 and CD168 are expressed on the cell membrane as hyaluronan receptors.[15] Hyaluronic acid directly stimulates the surrounding cells via this receptor, causing fibroblast migration and proliferation.

Activated fibroblasts produce collagen fibers and create lattice structures that contribute to morphological stability. However, as shown in a previous report[17] and our unpublished data, not all HAFs work as a scaffold. Currently available HAFs have a variety of viscosities and elasticities. According to a study of the biochemical characteristics of various HAFs[18], the Juvederm Vista ULTRA PLUS used in this study has moderate viscosity and elasticity. Kim et al. [26] report that HAFs with moderate viscosity act as a scaffold most effectively, although further investigations are necessary to clarify what ranges of viscosity and elasticity of HAF act as an adequate scaffold. The ideal fillers display no antigenicity, no toxicity, and no inflammatory properties. Long-term morphological stability is also ideal. Therefore, the properties that contribute to an effective scaffold in vivo may be important characteristics for ideal fillers.

CONCLUSIONS

Subcutaneous HAF injected as a bolus resulted in the migration of

fibroblasts and the formation of connective tissue to create lattice structures within the HAF. The HAF-injected space was gradually replaced by autogenous tissues comprising fibroblasts, collagen fibers, blood vessels, and adipocytes. This study reveals that HAF has bio-stimulatory effects that allow for maintenance of the primary volume of the HAF-injected space for 64 weeks after injection. A long-lasting effect of HAF can be expected when it is subcutaneously injected as a bolus that acts as a scaffold.

REFERENCES

1. Olenius M. The first clinical study using a new biodegradable implant for the treatment of lips, wrinkles, and folds. *Aesthetic Plast Surg*. 1998; 22: 97-101.
2. Brandt FS, Cazzaniga A. Hyaluronic acid gel fillers in the management of facial aging. *Clin Interv Aging.* 2008; 3: 153-159.
3. Carruthers JD, Glogau RG, Blitzer A. Advances in facial rejuvenation: botulinum toxin type a, hyaluronic acid dermal fillers, and combination therapies consensus recommendation. *Plast Reconstr Surg.* 2008; 121: 5S-30S.
4. Humphrey CD, Arkins JP, Dayan SH. Soft tissue fillers in the nose. *Aesthet Surg J.* 2009; 29: 477-484.
5. Cattin TA. A single injection technique for midface rejuvenation. *J Cosmet Dermatol.* 2010; 9: 256-259.
6. Becker M, Balagué N, Montet X, Calmy A, Salomon D, Toutous-Trellu L. Hyaluronic acid filler in hiv-associated facial lipoatrophy: evaluation of tissue distribution and morphology with MRI. *Dermatology*. 2015; 230: 367-374.
7. Choksi AN, Orringer JS. Linear morphea-induced atrophy treated with hyaluronic acid filler injections. *Dermatol Surg*. 2011; 37: 880-883.
8. Eastham AB, Liang CA, Femia AN, Lee TC, Vleugels RA, Merola JF. Lupus erythematosus panniculitis-induced facial atrophy: Effective treatment with poly-L-lactic acid and hyaluronic acid dermal fillers. *J*

Am Acad Dermatol. 2013; 69: e260-262.

9. Mashiko T, Mori H, Kato H, et al. Semipermanent volumization by an absorbable filler: Onlay injection technique to the bone. *Plast Reconstr Surg Glob Open*. 2013; 1: e4-e14.
10. Bennett R, Taher M. Restylane persistent for 23 months found during Mohs micrographic surgery: A source of confusion with hyaluronic acid surrounding basal cell carcinoma. *Dermatol Surg.* 2005; 10: 1366-1369.
11. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol*. 2000; 182: 311-322.
12. Bichakjan CK, Johnson TM. Anatomy of the skin. In: Shan R. Baker, eds. *Local Flaps in Facial Reconstruction.* 2nd ed. Philadelphia: Mosby; 2007: 3-13.
13. Goa KL, Benfield P. Hyaluronic acid. A review of its pharmacology and use as a surgical aid in ophthalmology, and its therapeutic potential in joint disease and wound healing. *Drugs*. 1994; 47: 536-566.
14. Viola M, Vigetti D, Karousou E, et al. Biology and biotechnology of hyaluronan. *Glycoconj J.* 2015; 32: 93-103.
15. Fraser JR, Laurent TC, Laurent UB. Hyaluronan: Its nature, distribution, functions and turnover. *J Intern Med.* 1997; 242: 27-33.
16. Fernandez-Cossio S, Catano-Oreja MT. Biocompatibility of two novel dermal fillers: histological evaluation of implants of a hyaluronic acid filler and a polyacrylamide filler. *Plast Reconstr Surg.* 2006; 117:

1789-1796.

17. McCleave MJ. Is breast augmentation using hyaluronic acid safe? *Aesthetic Plast Surg.* 2010; 34: 65-68.

18. Sundaram H, Cassuto D. Biophysical characteristics of hyaluronic acid soft-tissue fillers and their relevance to aesthetic application. *Plast Reconstr Surg* 2013; 132: 5S-21S.

19. Paliwal S, Fagien S, Sun X, et al. Skin extracellular matrix stimulation following injection of a hyaluronic acid-based dermal filler in a rat model. *Plast Reconstr Surg*. 2014; 134: 1224-1233.

20. Wang F, Garza LA, Kang S, et al. In vivo stimulation of de novo collagen production caused by cross-linked hyaluronic acid dermal filler injections in photodamaged human skin. Arch Dermatol. 2007; 143: 155-163.

21. Turlier V, Delalleau A, Casas C, et al. Association between collagen production and mechanical stretching in dermal extracellular matrix: In vivo effect of cross-linked hyaluronic acid filler. A randomized, placebo-controlled study. *J Dermatol Sci.* 2013; 69: 187-194.

22. Quan T, Wang F, Shao Y, et al. Enhancing structural support of the dermal microenvironment activates fibroblasts, endothelial cells, and keratinocytes in aged human skin in vivo. *J Invest Dermatol.* 2013; 133: 658-667.

23. Hom DB, Goding GS. Skin flap physiology. In: Shan R. Baker, eds. *Local Flaps in Facial Reconstruction.* 2nd ed. Philadelphia: Mosby;

2007: 15-30.

24. Mast BA, Diegelmann RF, Krummel TM, Cohen IK. Hyaluronic acid modulates proliferation, collagen and protein synthesis of cultured fetal fibroblasts. *Matrix.* 1993; 13: 441-446.

25. Greco RM, Iocono JA, Ehrlich HP. Hyaluronic acid stimulates human fibroblast proliferation within a collagen matrix. *J Cell Physiol.* 1998; 177: 465-473.

26. Kim DY, Namgoong S, Han SK, et al. Optimal Viscosity and Particle Shape of Hyaluronic Acid Filler as a Scaffold for Human Fibroblasts. *J Craniofac Surg*. 2015; 26: 1534-1538.

FIGURE LEGENDS

Fig. 1. HAF immediately following injection.

(*Above*) Macroscopic image captured immediately after a subcutaneous bolus injection of HAF into the back of a rat. (*Below*) Tissue sample stained with Alcian blue immediately after a subcutaneous bolus injection of HAF into the back of a rat. HAF is stained deep blue, and its presence is confirmed as a mass under the panniculus carnosus. Scale Bar = 5 mm.

Fig. 2. Timeline of HAF morphology and intensity as observed by MRI.

(*Above, left*) HAF was identified as a group of high signal areas between the skin and the muscle layer. (*Above, center*) After 4 weeks, the volume increased, the height diminished, and the shape flattened. (*Above, right*) Almost no change occurred between weeks 4 and 8. (*Below, left and center*) From 16 to 32 weeks, the intensity of the hyaluronic acid clearly declined and became heterogeneous. (*Below, right*) After 64 weeks, the intensity of the hyaluronic acid had declined further. Scale bars = 10 mm.

Fig. 3. Changes in the volume, height, and width of HAF over time as evaluated by MRI.

(*Above*) Volume, (*Center*) height, and (*Below*) width of HAF over time is shown. The mean value immediately following HAF injection (week 0) was used as 100%. Asterisks above graph indicate comparison to baseline (week

0). The asterisk together with a bar below the graph indicates a significant difference between the two time points at either end of the bar (*P < 0.05).

Fig. 4. Cell proliferation over time as indicated by HE stain and immunohistochemical staining of Ki67.

(*Above*) Autogenous-tissues, such as fibroblasts and adipocytes, are induced over time within the HAF-injected space. Proliferation of fibroblasts can be observed after 4 weeks and lattice structures have been formed. Scale bars = 500 μm. (*Below*) Change in the number of Ki67-positive cells with time. The mean value at each time point was calculated from sixteen random, high-magnification microscopic images. The number of Ki67 positive cells at 4-16 weeks was significantly higher than at baseline (week 0) (*P < 0.05).

Fig. 5. Histological findings of EVG staining and immunostaining of type III collagen.

(*Left column*) EVG-stained tissue samples demonstrate the presence of a blood vessel surrounded by collagen and smooth muscle (*arrowheads*) 4 weeks after HAF injection. (*Right column*) Immunostained tissue section shows that type III collagen was present in the connective tissues. Scale bars = 100 μm

Fig. 6. Proportion of collagen fibers in the HAF-injected space.

From weeks 0 to 8, the number of collagen fibers in the HAF rapidly

increased (by approximately 20%) and still occupied 15% of the HAF area, on average, at 64 weeks after HAF injection. (*P < 0.05; **P < 0.01)

Supplemental Digital Content 1. Immunohistochemical staining of Ki67 as an indicator of cellular proliferation

Specimen section at 4 weeks after HAF injection. Arrowheads indicate Ki67-positive cells. Scale bar = 50 μm

Supplemental Digital Content 2. Capsule formation around HAF over time. Arrowheads indicate the lateral boundary of the HAF-injected space. The lateral boundary is suitable for observing the capsule formation. The capsule-like membrane at week 0 is not a true capsule, but rather a structure formed by the compression of subcutaneous tissue during HAF injection. A capsule was detected at 4 weeks and later. Scar bars = 100μm

Supplemental Digital Content 3. Evaluation of tissue samples for calcification using von Kossa staining.

Tissue section 64 weeks after HAF injection. The arrowheads indicate the boundary of the HAF-injected space. No sections showed calcification within or around HAF at any of the time points. Scale bar = 100 μm.