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An injectable heparin-conjugated hyaluronan scaffold for local delivery of TGF- β 1 promotes successful chondrogenesis

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Abstract

Cartilage lacks basic repair mechanisms and thus surgical interventions are necessary to treat lesions. Minimally-invasive arthroscopic procedures require the development of injectable biomaterials to support chondrogenesis of implanted cells. However, most cartilage tissue engineering approaches rely on pre-culture of scaffolds in media containing growth factors (GFs) such as TGF- β 1, which are crucial for cartilage formation and homeostasis. GFs media-supplementation is incompatible with injectable approaches and led to a knowledge gap about GFs optimal dose and release profile to achieve chondrogenesis. This study aims to determine the initial amount and release kinetics of TGF- β 1 bound in an engineered GAG hydrogel, to promote cartilaginous matrix production in absence of TGF- β 1 media-supplementation. We show that heparin, a GAG known to bind a wide range of GFs, covalently conjugated to a hyaluronan hydrogel, leads to a sustained release of TGF- β 1. Using this heparin-conjugated hyaluronan hydrogel, a range of loaded TGF- β 1 doses from 0.25 to 50 ng/scaffold is tested on encapsulated chondroprogenitor cells for viability, proliferation and cartilaginous matrix deposition. We unravel an increase of all parameters together with the initially entrapped dose of GF up to 20 ng/scaffold, with 5 ng TGF- β 1/scaffold being the minimum dose to promote chondrogenesis. We also demonstrate the necessity of a sustained release of TGF- β 1, as no

matrix deposition is observed upon a burst release. In conclusion, our biomaterial loaded with an optimal initial dose of 5 ng/scaffold TGF- β 1 is a promising injectable scaffold for cartilage repair, with potentially increased safety due to the low GF dose, locally administered.

Keywords: hyaluronan; Heparin; chondroprogenitors; TGF- β ; cartilage; injectable

Graphical abstract:

1. Introduction

Full-thickness focal chondral defects are common among athletes, with an overall prevalence of 36% [1]. However, due to the cartilage low cellularity and lack of vasculature, this tissue has a well-known poor self-healing capacity [2]. Treatment of cartilage lesions therefore requires a surgical procedure [3]. The gold standard is Autologous Chondrocyte Implantation (ACI), which relies on the isolation of the patient's own chondrocytes from a low-loadbearing region of the joint, followed by an expansion phase *in vitro* and later re-implantation in the defect [4, 5]. Although this technique has provided good clinical results, especially for lesions larger than 5 cm² [6, 7], it is associated with two main drawbacks. Firstly, the graft requires a long maturation time to reach a hyaline-like quality of repaired tissue [8]. Secondly, the procedure is invasive as it includes two steps; the second being an invasive arthrotomy associated with long rehabilitation periods. The invasiveness of the ACI technique has motivated the development of injectable materials, which could be delivered via arthroscopy [9]. Such materials include the commonly used fibrin glue (Tisseel from Baxter) or Novocart Inject from Tetec, which has reached the stage of clinical trials [10]. However, although injectable, these biomaterials do

not have the intrinsic property of stimulating chondrogenesis; hence the need for smart biomaterials containing bioactive cues such as growth factors (GF) to guide the re-differentiation.

GFs are soluble proteins acting in a paracrine or autocrine manner to stimulate cell growth [11]. In the field of cartilage repair, they are used either supplemented in the culture medium or present in a 3D matrix with or without encapsulated cells (condition referred to as “loaded”) [14, 15, 16]. TGF- β signaling has been demonstrated to be critical for cartilage morphogenesis [16, 17, 18]. TGF- β 1, the predominant isoform in cartilage [19] is involved in cartilage embryonic development [16, 18, 20], maturation at the postnatal stage, and cartilage homeostasis during adulthood, [18, 22, 23]. TGF- β s have consequently been extensively used to stimulate chondrogenesis for cartilage tissue engineering [24 - 26].

In rodents, doses as high as 600 ng (200 ng injected three times in 4 days to stimulate cartilage repair in an arthritis model) [31] or even 1 μ g of TGF- β 1 [32] have been injected in the knee joint in the study to study the role of TGF- β 1 in the pathophysiology of arthritis. Such high doses have been reported to cause pathological effects including inflammation [32], off-target effects including synovial hyperplasia and osteophyte formation [33, 34]. Moreover, TGF- β 1 is a potent molecule active in the picomolar range *in vivo* and this activity at low doses is linked to a risk of tumorigenesis due to overstimulation of proliferation, upon exposure to supraphysiological doses [11, 35]. The side effects of TGF- β 1 support the need for a local delivery of GFs in the lesion site and more appropriately loaded into a scaffold.

A low dose of GF with a sufficiently long exposure to the encapsulated cells in the site of injury are thus two desired features for use in the clinics, which would justify the need for scaffolds promoting a sustained release of the GF. Indeed, systems where the GF is unbound and quickly diffuses into the synovial fluid (condition referred to as “burst release”) may not provide sufficient chondrogenesis stimulation. Several approaches have been developed to achieve a sustained release from hydrogels [36, 37]. Loading strategies more specific to GFs [38 - 41] and more precisely to TGF- β 1 [25] have been reviewed. Among these strategies,

sequestration of growth factors by glycosaminoglycans (GAGs) is an interesting approach as it mimics the way GFs are bound to the sulfated polysaccharides of the extracellular matrix (ECM) in native cartilage tissue [42, 43]. Chondroitin sulfate has been covalently incorporated in a covalently-crosslinked polyethylene glycol network and was shown to allow a slow release of BMP-2, which could be tuned with the ratio of chondroitin sulfate in the system [44]. Similarly, heparan sulfate binds with high affinity to a myriad of proteins, including GFs, to regulate their availability and signaling [45]. Its hypersulfated form, heparin, is extensively used in the clinics for its anti-thrombotic properties and has also interestingly been shown to stabilize the proteins it binds to such as FGF2, by protecting FGF2 against acidic or heat treatment [46] and by increasing FGF2 shelf-life during storage [47]. Among the heparin-binding proteins is TGF- β 1 [48, 49]. Heparin has consequently been used for drug delivery purposes in the context of tissue engineering [50, 51], either incorporated as a semi-interpenetrated polymer within a crosslinked network [52, 53] or covalently bound to a polymer backbone. The covalent binding of heparin was achieved in different ways, by grafting tyramine [54], methacrylate or thiols [55] or maleimides [56] moieties to the heparin chains. All these studies using covalently-bound heparin aimed at enhancing the biological properties of the hydrogels. However, despite mentioning the capacity of heparin to bind TGF- β , they all have used TGF- β supplemented within the medium when investigating chondrogenesis of encapsulated cells.

Heparan sulfate is present at the stage of embryonic and fetal development, which provides an interesting feature for its use in combination with young chondrocytes. Chondrocytes from infant [57, 58] or fetal [59] donors have been investigated due to their higher proliferation capacity and better retention of chondrogenic phenotype during expansion compared to adult chondrocytes that drift to a more fibroblastic phenotype [60, 61]. More particularly, human chondroprogenitor cells (hCCs) from fetal origin have shown great promises for cartilage tissue engineering. They were used in combination with TGF- β , either seeded on commercially available collagen sponges [62] or encapsulated in a hyaluronan-based hydrogel, enzymatically crosslinked with transglutaminase factor XIII (also referred to as HA-TG, for Hyaluronan-Transglutaminase [63]) [64]. The potential of the combination of HA-TG and hCCs

for cartilage repair was investigated *in vitro* with TGF- β 3 medium supplementation [64]. With a view to developing an injectable therapy for cartilage repair, administered via arthroscopy without any pre-culture step, the GF should be injected together with the cells for maturation of the construct *in vivo*. This is the limitation to translation of the majority of *in vitro* studies focused on cartilage regeneration strategies, which use GF medium supplementation whether it is to determine the GFs nature and dose [65, 66] or exposure time [67] for the best chondrogenesis stimulation.

To our knowledge, no other study has focused on using a biomaterial allowing a sustained release of a wide range of molecules (in the case of this study, heparin-binding molecules) as a screening platform to better understand the role of a given molecule in the complex process of chondrogenesis. In this study, we characterized a strategy to load TGF- β 1 in the HA-TG system, to avoid a burst release. Since hCCs have not been extensively studied with regards to their response to TGF- β 1, heparin-conjugated HA-TG was used as a tool to screen TGF- β concentrations to better understand the correlation of the GF dose and the quality of the generated tissue engineered product. We further assessed whether the assumption that a sustained rather than a burst release is the most effective way of stimulating hCCs chondrogenesis. **Figure 1.**

2. Material and methods

2.1 HA-TG and HA-TG/Hep-TG synthesis and characterization

2.1.1. Synthesis

The synthesis of HA-TG was done as previously described [63, 64], by covalently grafting high molecular weight Hyaluronan (Lifecore) with transglutaminase peptides, either a Lysine-donor peptide (FKGG-ERCG, HA-Lys) or a Glutamine-acceptor peptide (NQEVSPL-ERCG, HA-Gln). The synthesis of Hep-TG was performed using the same protocol. 100 mg (in other terms 0.168 mmol of carboxylic acid moieties, considering a molecular weight of 593 g/mol for a disaccharide repeat unit) of Heparin (Sigma-Aldrich) was dissolved in milliQ water. Once solubilized, 1.5 mmol of MES hydrate (Applichem), 0.025 mmol of 3,3"-Dithiobis(propanoic dihydrazide) (Frontier Scientific) and 0.05 mmol of 1-Ethyl-3-(3-

dimethylaminopropyl)carbodiimide, to obtain 15% substitution of the carboxylic acid moieties. After leaving overnight to react (> 16 hours), the solution was reduced by addition of 0.15 mmol of tris(2-carboxyethyl)phosphine (Fluorochem) for 8 hours and subsequently dialysed against acidified miliQ water after addition of 20 mmol of NaCl (24 hours, 6 water changed). The thiolated heparin was then added dropwise into a Triethanolamine buffer solution (0.395 ml of Triethanolamine in 8 ml of miliQ water, pH 8) containing 2.5 mmol of Divinylsulfone (Sigma), left to react for 2 hours, supplemented with 70 mmol of NaCl and dialysed against water. The grafting of vinylsulfone moieties was confirmed with NMR (Fig. A). In the last step, the pH was adjusted to 8, the vinylsulfonated heparin was bubbled with nitrogen to avoid oxidation of the peptides, and a 1.5-fold excess (0.04 mmol) of Glutamine-donor peptide (NQE QVSPL-ERCG) was added. The full substitution of vinylsulfone moieties by the peptides was confirmed with NMR with the absence of vinylsulfone peak (Fig. A). Starting material (hyaluronan and heparin) and final products (HA-TG, Heparin-TG) were tested for endotoxins using the QUANTI-Blue™ test from InvivoGen, according to manufacturer's protocol. Results were negative (lower than the detection limit) for all final products (Fig. A).

2.1.2. HA-TG/Hep and HA-TG/Hep-TG gelation

To make HA-TG/Hep and HA-TG/Hep-TG, polymers were resuspended in a solution of "TBG" buffer (100 mM Glucose, 50 mM Tris base, 50 mM CaCl₂, balanced to pH 7.6), a double-concentrated solution of HA-TG (4% w/v) was mixed with a double-concentrated solution of heparin or heparin-TG (0.2% w/v). Of note, the ratio HA-Lys / HA-Gln was kept at 50% due to the minimal amount of Hep-Gln added in the system (20 times less than HA-TG). In case of growth factor loading, the appropriate amount of a highly concentrated stock solution (100 µg/ml) of TGF-β1 (Peprotech AF-100-21C) was added to the heparin vial for at least 15 minutes to reach equilibrium binding, prior to the mixing of HA-TG and heparin. Gelation was triggered by adding of Thrombin (Baxter, 1/40th of the total volume of gel) and Transglutaminase FXIII (Fibrogammin from CSL Behring, 1/20th of total volume of gel) and pipetting up and down before casting 25 µl in PDMS hollow cylindrical molds of 1-2 mm high

and 4 mm internal diameter. The casted mixture was kept 15 minutes at 37°C before addition of culture medium.

2.2. Rheometry

The gel precursors were mixed to obtain a total volume of 50 µl, without cells. Gelation was triggered by addition of 1.25 µl of thrombin and 2.5 µl of FXIII and stirring for 3 seconds. Of this gelling mixture, 40 µl was loaded on an Anton Paar MCR 301 rheometer. The rheometer was prepared with a 20 mm plate-plate geometry, a metal floor pre-heated to 37°C, a surrounding of wet tissue to create a humidified environment. The probe was lowered to 0.1 mm and the measurement was made at 1 Hz with 4% strain.

2.3. Cell encapsulation and live imaging

2.3.1. hCCs expansion and encapsulation

hCCs were isolated at the Lausanne University Hospital (CHUV) in the Unit of Regenerative Therapy as previously described (Ethics from Centre Hospitalier Universitaire Vaudois Ethics Committee Protocol No.62/07 and registered under the Federal Transplantation Programme complying with the law and Biobank procedures) [59, 64]. A working cell bank of hCCs at passage 2 was prepared for the study. Frozen vials were thawed and in Dubelcco's modified Eagle's medium 41966 (DMEM, Gibco) supplemented with 10% v/v FBS (Gibco), 1% (2 mM) L-glutamine (Gibco) and 1% (100 U/ml) Penicillin-Streptomycin (Gibco). After a one-week expansion, the cells were trypsinized (i.e. used at passage 3) and mixed with the gel precursors at a concentration of $15 \cdot 10^6$ cells/ml. After gelation as described above, the scaffolds were cultured in "control medium", consisting of DMEM 31966 supplemented with 10 µg/ml Gentamicin (Gibco), 50 µg/ml L-ascorbate-2-phosphate, 40 µg/ml L-proline, 1% ITS+ Premix (Corning), at 37°C, 5% v/v CO₂ with 3 changes of medium per week. Only in the "Med." condition, 10 ng/ml of TGF-β1 was added in the culture medium.

2.3.2. Live dead staining

Cellular viability was investigated using a standard Live Dead assay, according to manufacturer's instructions. Briefly, the gels after *in vitro* culture were immersed in a solution of DMEM (Gibco, 31966) with 2µM Calcein AM, 20 µM propidium iodide (Thermofisher) and 10 µg/ml Hoechst, for 1 hour in the incubator (37°C, 5% CO₂, 95% humidity). After 3 washes in pre-warmed PBS, samples were imaged with a ZEISS AxioObserver inverted epifluorescence microscope (ApoTome.2) at 10x magnification, using the ZEN software. All

quantifications were performed with Fiji software. The viability was calculated by dividing the number of living cells by the total number of cells (live + dead) and averaging the values of the triplicates.

2.4. Biochemical analysis

2.4.1. Samples preparation

The scaffolds were frozen at -80°C and lyophilized overnight. They were digested by adding 500 µl of a 270 µg/ml solution of papain from Papaya Latex (Sigma Aldrich) in a buffer containing 5 mM L-cysteine HCl, 50 mM sodium phosphate, 2 mM EDTA in miliQ water, with a pH adjusted to 6.3. The digestion was done at 60°C overnight with shaking (1000 rpm).

2.4.2. DNA quantification (Picogreen®)

The amount of DNA was quantified using a Quant-iT PicoGreen® dsDNA assay kit (Invitrogen) as per manufacturer's protocol. Briefly, the samples were pre-diluted 1:10 in TE buffer. 50 µl of this dilution was transferred in a 96-well plate and 50 µl of a Picogreen solution was added. The fluorescence was measured with a microplate reader (excitation 480 nm, emission 520 nm). The DNA content was noted as µg DNA/scaffold, normalized to day 0.

2.4.3. DMMB assay

The amount of GAGs was assessed using dimethylmethylene blue solution (21 mg of 1, 9-dimethyl methylene blue zinc chloride double salt in 5 ml absolute ethanol with 2 g sodium formate, further diluted in 800 ml miliQ water; pH was adjusted to 3 with concentrated formic acid the volume was topped to 1 L with miliQ water). 40 µl of the digested sample (1:200 dilution in papain buffer to obtain values within the linear range of the standard curve) were transferred into a 96-well plate and 100 µl of DMMB solution added to the wells. The absorbance at 595 nm was measured on a microplate reader and the corresponding amount extrapolated from a linear regression of a standard curve. This latter was obtained with either chondroitin 4-sulphate sodium salt from bovine trachea (Sigma-Aldrich, 0 to 50 µg/ml, used for scaffolds containing cells after 3 weeks of culture) or a solution of heparin or heparin-TG (0 to 100 µg/ml for heparin release experiments). GAG contents were reported per sample or

normalized to the day 0 background (important when comparing HA-TG and HA-TG/Hep-TG, considering the sulfates moieties of heparin).

2.5. Histological analysis

The gels were fixed in 4% formaldehyde for 1 hour and dehydrated by immersion in gradually increasing ethanol concentration baths. Paraffin impregnation was done in an automated processor (LogosJ, Milestone SRL) and the samples were embedded so that they can be cut coronally (one section showing the middle of the gel, top to bottom). 5- μ m thick sections were cut at the microtome, deparaffinized in xylene and rehydrated in ethanol baths of decreasing concentration. Immunohistochemistry stainings of collagen I and II included an antigen retrieval step (1200 U/ml of hyaluronidase (Sigma) for 30 minutes at 37°C), a blocking step with 5% Normal Goat Serum (NGS, Abcam) in PBS for 1 hour at room temperature. Rabbit anti human primary antibodies diluted in 1% NGS in PBS (collagen type II antibody: Rockland, 600-401-104, 1:200 / collagen type I antibody: Abcam, #ab138492, 1:1500) were applied overnight at 4°C. After rinsing and quenching of the endogenous peroxidase activity (1% H₂O₂ in water for 20 minutes), slides were incubated with a goat anti-rabbit IgG-HPR (Abcam, ab6721, 1:1500 dilution in 1% NGS in PBS) for 1 hour at room temperature. The DAB kit (Abcam) was used to reveal the antibodies (4 minutes), as per manufacturer's protocol. Staining with Safranin O was done in accordance with standard protocols. All stainings were followed by a dehydration step in increasing concentrations of ethanol and two baths of xylene before mounting the slides with a resin mounting media (Eukritt). Images were acquired using a Slide Scanner (Pannoramic 250 Flash II).

2.6. Gene expression with q-RT-PCR

After 21 days of culture, the scaffolds were snap frozen in liquid nitrogen and crushed in 1 ml of Trizol with pellet pestles (Thomas Scientific) and 200 μ l of chloroform. After 15 minutes incubation at RT, the tubes were spun 10 minutes at 12'000 g prior to total RNA extraction with a NucleoSpin® miRNA kit (Macherey-Nagel), according to manufacturer's protocol. The RNA concentration was quantified with a microplate reader Synergy H1 (BioTek Instruments) and only samples whose absorbance ratio 260:280 was between 1.9 and 2.1 were used for q-RT-

PCR experiments. 195 ng of RNA were reverse transcribed, in a 20 µl reaction volume and further processed as previously described in [62]. PCR amplification was performed with SYBR Green (Applied Biosystems) with 150 nM of both forward and reverse primers, which were designed across exon-exon junctions and used in previous studies [62, 64]. Each condition was tested in triplicates, with 2-3 readings per replicate. The analysis utilized the $2^{-\Delta\Delta C_t}$ method and results were normalized to a reference gene RPL13a. Cells at day 0 were chosen as the reference.

2.7. Unconfined compression testing

The mechanical testing was performed with a TA.XTplus Texture Analyser (Stable Microsystems) with a 500 g load cell and a 2 mm diameter cylindrical probe. The gels were subjected to a 0.2 g preload to ensure the contact between the gels and the probe. The gels were compressed to a final strain of 10%, at a loading rate of 0.01 mm/s. The E modulus (compression modulus, kPa) was determined as the slope of the linear part of the stress-strain curve.

2.8. Statistical analysis

All experiments were performed in triplicates (viability, gene expression, histology) or quadruplicates (compression test, biochemical assays) [exception of duplicates for the growth factor screen histology in Fig. C]. Two (biochemical assays) or three (viability, q-RT-PCR) technical replicates were read. Quantitative assays are reported as mean $\pm$ standard deviation of the replicates (each single point is the average of the 2 or 3 technical replicates for each sample). All statistical test were performed with Prism (GraphPad Software Inc., version 7.0.3). Comparisons of the different TGF- β 1 doses were done with one-way ANOVAs followed by Tukey's *post hoc* tests comparing each condition to the "M" (TGF- β 1 supplemented in the medium) condition. For a specific dose of TGF- β 1, comparisons between HA-TG and HA-TG/Hep-TG were done using an unpaired, two-tailed Student t test. Statistical significance was considered when $p < 0.05$ (noted "**", $p < 0.01$ denoted as "***" and $p < 0.001$ as "****", $p < 0.0001$ as "*****").

3. Results

3.1. Covalent binding of heparin to HA-TG allows a sustained release of TGF- β 1 and enhances chondrogenesis of hCCs

We first investigated whether the addition of heparin to sustain the release of a GF from HA-TG led to better retention of the GF when heparin was simply mixed with HA-TG (thus forming a polymer blend, “HA-TG/Hep”) or covalently bound (leading to a graft copolymer, “HA-TG/Hep-TG”).

An important feature of HA-TG is its rapid gelation onset and equilibration, which allows a possible administration via an arthroscopy probe and a polymerization *in situ* within the cartilage defect. To determine whether the addition of heparin in the HA-TG system modifies the gelation behavior, we characterized the gelation kinetics of HA-TG/Hep and HA-TG/Hep-TG with a rheometer. As previously described, HA-TG gelation reaches a plateau after 10 minutes. Hep-TG interfered with HA-TG crosslinking, without reinforcing the mechanical properties due to its small molecular weight. This observation was also reported in another system based on covalent crosslinking of heparin-tyramine to dextran-tyramine [54]. The delayed crosslinking and lower storage modulus after 50 minutes was supposedly due to the competition with the crosslinking of the gel precursors (the HA chains grafted with either a Glutamine-donor peptide or a Lysine-acceptor peptide), as suggested by the absence of crosslinking delay when unmodified heparin is added. This observation did not rule out the possibility to add Hep-TG to the system since the formation of a gel could still be observed (G' and G'' curves crossed after ~ 2 minutes) (Fig. 2A).

In a second step, we monitored the amount of heparin diffusing out of the gels over two weeks, using a biochemical DiMethyl Methylene Blue (DMMB) assay. As a control, we monitored the presence of GAGs released from HA-TG scaffolds, which was negative at all timepoints (data not shown). We observed a fast diffusion of heparin when it was not crosslinked, with $\sim 35\%$ of the initial loaded amount released from HA-TG/Hep scaffolds at the first investigated timepoint of 2 hours (Fig. 2B). On the other hand, covalent binding of heparin (Hep-TG) allowed better retention in the HA-TG/Hep-TG scaffolds ($\sim 6\%$ released after 2 weeks vs $\sim 70\%$ for unbound heparin, $p < 0.0001$).

Interestingly, both heparin incorporation strategies allowed a sustained release of TGF- β 1 as shown by the overlapping release curves (Fig. 2C). In absence of heparin, there was a clear burst release of TGF- β 1 from HA-TG scaffolds, as expected [38]. Indeed, 49 ± 2 % of the initially loaded amount diffused out after 2 hours and 75 ± 3 % already at the second time point (9 hours). At the end of the two-week experiment, the total amount of TGF- β 1 released from HA-TG gels was 97 ± 3 % of the initial amount, vs 60 ± 4 % upon addition of Hep-TG ($p < 0.0001$) and 60 ± 2 % for unbound heparin ($p < 0.0001$). Despite the same GF release profile upon incorporation of bound and unbound heparin to HA-TG, extracellular matrix (ECM) deposition by encapsulated hCCs was not promoted by addition of unbound heparin (Fig. 2D). We therefore chose HA-TG/Hep-TG for further investigations. **Figure 2.**

3.2. Covalent addition of heparin to HA-TG does not hinder the chondro-inductive properties of HA-TG

We first sought to characterize the capacity of HA-TG/Hep-TG in TGF- β 1-supplemented medium to stimulate proliferation of, and cartilaginous matrix deposition by hCCs after 21 days of culture. The goal was to confirm that HA-TG/Hep-TG did not impair the previously described chondro-inductive properties of HA-TG [64] and to validate the use of HA-TG/Hep-TG in TGF- β 1-supplemented medium as a control condition for a TGF- β 1 dose screening.

We observed similar viability (79 ± 3 % in HA-TG vs 78 ± 3 % in HA-TG/Hep-TG, $p = 0.56$) and proliferation (3.1-fold increase of DNA content compared to day 0 in HA-TG vs 2.8 in HA-TG/Hep-TG, $p = 0.07$) of hCCs in both scaffolds (Fig. 3B). The quantification of deposited GAGs showed less accumulation over 3 weeks upon addition of Hep-TG (1.6-fold increase compared to the background at d0 in HA-TG/Hep-TG vs 3.0 in HA-TG, $p = 0.01$). However, the gene expression level of the GAGs core protein, aggrecan, was similar in both conditions at day 21 ($p = 0.32$, Fig. 3C). Additionally, gene expression of collagens, both type I and II, was similar in both biomaterials ($p = 0.5$ for type I gene expression and $p = 0.08$ for type II) (Fig. 3D). We measured a lower compressive modulus at day 21 for HA-TG/Hep-TG constructs (76 ± 4 kPa) compared to HA-TG ones (88 ± 5 kPa, $p = 0.009$, Fig. 3E), which could partly be explained by the rheological observations (Fig. 2A). Indeed, rheological data highlighted a lower storage

modulus still after 50 minutes of crosslinking (540 ± 60 Pa for HA-TG vs 210 ± 10 Pa for HA-TG/Hep-TG, $p=0.0006$ after unpaired). However, the stiffness of 76 kPa measured after 21 days, is within the range of compression moduli obtained after 3 weeks of culture of hCCs encapsulated in 1% (150 kPa) and 2% (25 kPa) HA-TG in TGF- β 3 supplemented medium [64]. We therefore concluded that HA-TG/Hep-TG is suitable to pursue the development of an injectable cartilage tissue engineering approach. We used this biomaterial as a platform to screen TGF- β 1 concentrations, to find a range of initially loaded doses of the GF that stimulates chondrogenesis *in vitro* and to further understand the correlation between this initial dose and hCCs phenotype. **Figure 3.**

3.3. hCCs show a dose-response to the initially loaded dose of TGF- β 1 in terms of viability and proliferation

We quantified the effect of the initially loaded dose of TGF- β 1 on the viability and the proliferation of hCCs after 3 weeks of culture in a defined, serum- and GF-free culture medium. We compared the values to the previously described condition of 10 ng/ml TGF- β 1 supplementation in medium ("M" condition). Quantification of Live-dead images showed that the more TGF- β 1 initially loaded in HA-TG/Hep-TG, the higher the viability. Indeed, the viability after the 3 weeks of culture in serum- and TGF- β 1-free medium became significantly lower than in the TGF- β 1 supplemented medium, from a dose of 1.25 ng/scaffold ($\sim 50\%$ vs 80% , $p=0.03$) and under ($p<0001$ for 0.25 and 0 ng/scaffold) (Fig. 4B). The lowest dose, 0.25 ng/scaffold, did not prevent cell death as the viability was similarly low as in scaffolds not loaded with TGF- β 1 ($\sim 20\%$). Interestingly, we could also observe a different cell morphology and distribution according to the administration route (medium vs loaded) and the initial dose of TGF- β 1. When TGF- β 1 was supplemented in the medium, some cells adopted an elongated shape, radially oriented towards the surface of the gel (Fig. 4D, white arrow), which did not happen when TGF- β 1 was loaded in the gels. In addition, at 1.25 ng/scaffold (equivalent to 50 ng/ml), we noticed that the cells were not homogeneously distributed, but rather proliferated in clusters (white arrow and dashed circled cluster, Fig. 4D).

Regarding the effect of TGF- β 1 dose on hCCs proliferation, no dose-response could be observed, but rather a threshold at 1.25 ng/scaffold ($\sim$ 2-fold increase of the DNA content compared to d0), under which the proliferation was significantly impacted as compared to the condition of TGF- β 1-supplemented medium ($\sim$ 3-fold increase, $p < 0.0001$) (Fig. 4C). In other words, TGF- β 1 loaded in HA-TG/Hep-TG promoted proliferation of hCCs, to a similar extent as continuous supplementation in the culture medium, from 5 ng/scaffold (equivalent to 200 ng/ml) onwards. **Figure 4.**

3.4. hCCs produce more fibrocartilage and hypertrophic ECM compounds in presence of high amounts of TGF- β 1

Following the promising viability and proliferation behavior of hCCs upon TGF- β 1 loading, we further investigated the efficiency of the loading to stimulate cartilaginous ECM production. At 1.25 ng/scaffold, the ECM was deposited pericellularly, and no staining was detected for lower loaded dosages. From 5 ng TGF- β 1/scaffold and higher, the tissue obtained after *in vitro* culture displayed some hyaline-like features, notably the round shape of the cells, the homogeneously deposited ECM and the intense staining for collagen type II (Fig. 5A). GAGs were also produced as shown by the strong Safranin O staining, with a gradually lower staining intensity with the decrease in TGF- β 1 loaded amount, suggesting a dose-response of GAG production according to the loaded dosage of the GF (Fig. 5F). GAGs quantification confirmed the histological findings, further highlighting increased amounts of GAGs in scaffolds loaded with 50 ng ($660 \pm 74 \mu\text{g}$, $p = 0.006$), 20 ng ($700 \pm 36 \mu\text{g}$, $p = 0.001$) and 5 ng ($600 \pm 85 \mu\text{g}$, $p = 0.04$) of TGF- β 1/scaffold, as compared to the scaffolds cultured with TGF- β 1 in the medium ($422 \pm 160 \mu\text{g}$) (Fig. 5H).

Histological staining additionally displayed features closer to fetal, rather than adult hyaline cartilage tissue, such as a high chondrocytes density and the absence of zonal distribution of chondrocytes [69], which were also reported in ACI grafts 2 to 9 years after surgery [70]. Moreover, collagen type I, present at early stages of cartilage development [71] but also known as a marker of fibrocartilage, was deposited in a dose-dependent manner as shown by the increasing intensity of the staining and the more homogeneous spatial distribution together with the loaded amount of TGF- β 1 (Fig. 5A). Collagen type X, produced during development

by chondrocytes undergoing hypertrophy, was also detected by immunohistochemistry for all doses of TGF- β 1, in seemingly higher amounts when TGF- β 1 was loaded in the gel instead of supplemented in the medium (Fig. 5A).

We measured the gene expression of collagens at day 21. From 5 ng TGF- β 1/scaffold onwards, collagen type II was significantly higher expressed than in the medium supplementation condition ($p=0.01$ for 50 ng/scaffold, $p=0.0007$ for 20 ng/scaffold, $p=0.007$ for 5 ng/scaffold). Interestingly, 20 ng/scaffold yielded the best and most reproducible collagen II gene expression increase, suggesting that “the more the better” does not apply for TGF- β 1 loading (Fig. 4B). Additionally, loading of TGF- β 1 into HA-TG/Hep-TG led to lower Collagen type I gene expression as compared to day 0, contrary to medium supplementation that was associated with a 6-fold increase ($p<0.0001$, Fig. 5C). Collagen type X gene expression followed the same trend as for collagen type I. Indeed, all conditions with loaded TGF- β 1 led to a reduced expression as compared to medium-supplemented TGF- β 1 ($12'300 \pm 3'000$ with TGF- β 1 in medium vs 180 ± 100 / $1'300 \pm 600$ / 30 ± 6 / 14 ± 8 -fold increase for doses from 50 to 1.25 ng/scaffold respectively, $p<0.0001$ for all conditions, Fig. 5D).

The production of ECM led to increased compression moduli of the scaffolds from 5 ng/scaffold and higher (Fig. 5E). The initial loading of 5 ng/scaffold resulted in a similar stiffness (77 ± 22 kPa) as the one obtained with TGF- β 1 medium supplementation (76 ± 4 kPa, $p=0.99$). Both 50 ng/scaffold (102 ± 14 kPa, $p=0.03$) and 20 ng/scaffold (118 ± 19 kPa, $p=0.0005$) allowed to reach higher compression moduli than medium supplementation. The highest compressive modulus was achieved by loading 20 ng/scaffold, which further strengthens the idea that TGF- β 1 efficiency reaches a plateau at these high doses.

In summary, TGF- β 1 stimulated hCCs survival, proliferation and cartilaginous matrix deposition, in a dose responsive manner, with a threshold at 5 ng/scaffold (200 ng/ml) and a plateau above 20 ng/scaffold (800 ng/ml). We thus considered the dose of 20 ng TGF- β 1/scaffold to test the hypothesis that a sustained release would lead to enhanced chondrogenesis, as opposed to a burst release. **Figure 5.**

3.5. The sustained release kinetics of TGF- β 1 is necessary for hCCs survival, proliferation and ECM production

Little is known about the required timing of TGF- β 1 exposure to achieve chondrogenesis, and a boost of TGF- β 1 resulting from a burst release could already be sufficient [25, 72, 73].

The Live-dead staining revealed that despite the burst release from HA-TG, the viability of encapsulated hCCs in HA-TG ($53.3 \pm 9.4\%$) remained similar to the one in HA-TG/Hep-TG ($64.8 \pm 5.9\%$, $p=0.15$) upon exposure to an initial loaded amount of 20 ng TGF- β 1/scaffold. However, proliferation was significantly affected by the burst release (1.2 ± 0.5 DNA fold increase compared to d0 in HA-TG, vs 2.6 ± 0.1 in HA-TG/Hep-TG, $p=0.002$), as already visible in Live-dead stainings displaying lower cell density in HA-TG (Fig. 6B). In other terms, an initial administration of TGF- β 1 is sufficient for cell survival but an extended exposure to the GF is needed for proliferation.

The stiffness of the HA-TG scaffolds (6.2 ± 0.8 kPa) was also significantly lower than HA-TG/Hep-TG (117.7 ± 18.7 kPa, $p<0.0001$) after 21 days. This correlated with the absence of both GAGs and collagens deposition in HA-TG scaffolds (Fig. 6C-D) as observed on histological stainings. The quantification of GAGs deposition was consistent with histology findings, showing a 2.6 fold increase in the case of a sustained release, vs 1.2 with a burst release ($p=0.002$). Of note, this increase was due to the proliferation of the cells, as the normalization of GAGs amounts to DNA amounts did not show a significant difference ($p=0.053$). The stimulation of GAGs production appeared to be long-lasting since still at day 21, hCCs display a 9.2 ± 1.8 -fold increase of aggrecan gene expression compared to day 0, vs 0.4 ± 0.5 in case of burst release ($p=0.001$). The observations were the same for collagen type II, with a 39-fold increase of its gene expression in scaffolds with a sustained TGF- β 1 release ($p<0.0001$). Inversely, the burst release correlated with a 12-fold increase of collagen type I expression compared to the sustained release system ($p=0.014$). TGF- β 1 therefore requires a sustained release to successfully promote matrix deposition and to maintain the chondrogenic phenotype of the cells. Indeed, none of the doses of TGF- β 1 tested in this study could promote chondrogenesis upon a burst release (Fig. D). **Figure 6.**

4. Discussion

The present study takes place in the context for the search for ACI improvements, notably through the combination of potential off-the-shelf progenitor cells and smart biomaterials [41] together with stimulating factors [74]. There is a strong interest for injectable scaffolds in the field of cartilage tissue engineering [9], which could represent a manner to provide the patients with a non-invasive surgical approach. For an injectable, this requires development of biomaterials capable of binding GFs to stimulate the regeneration process *in vivo*. In this study, we report the enhancement of a previously described biocompatible, injectable and chondro-inductive biomaterial, HA-TG, in order to add some GF-binding moieties. We report three main findings. Firstly, chemically-modified heparin (Hep-TG) kept its GF binding property as seen by TGF- β 1 sustained release and could promote matrix production unlike the unbound heparin. Secondly, we found a dose-response for the stimulation of proliferation and matrix production, with 5 ng of TGF- β 1/scaffold (i.e. 200 ng/ml) being the lowest efficient dose and an increasingly fibrocartilaginous composition being observed with higher doses of TGF- β 1. Thirdly, we found that a sustained release is necessary to stimulate cartilaginous matrix deposition, even with a high initial loaded dose of TGF- β 1.

In the first instance, we investigated the proper way to introduce heparin to the system, namely either semi-interpenetrated in or covalently bound to HA-TG. An important finding of this study is that the sustained release is not the only critical parameter for chondrogenesis as HA-TG/Hep, despite its capacity to sustain TGF- β 1 release, did not lead to ECM deposition by hCCs. The persistence of a certain amount of sulfates moieties therefore seems to be an important factor for cartilage regeneration, as suggested by previous studies reporting enhanced chondrocytes redifferentiation upon encapsulation in 3D matrices containing heparin [54-56]. The presence of sulfates is not sufficient *per se*, because hCCs in HA-TG/Hep-TG cultured in serum- and TGF- β 1-free medium did not display any ECM deposition (Fig. 4A, F). A possible explanation for the necessity of the persistence of some sulfates moieties in combination with the presence of TGF- β 1 is that heparin binds to the newly produced ECM after an initial stimulation of chondrogenesis by the GF, as heparin is known to bind collagens and other ECM proteins [75]. These results could also be linked to the capacity of heparin to

bind to the GF in the direct surrounding of the scaffolds, therefore prolonging the effect of this GF. This hypothesis is supported by the observation in the recently published study of Cavalli et al. [68] of a Collagen type II pericellular deposition by infant chondrocytes in HA-TG/Hep-TG scaffolds in an ectopic mouse model, even in absence of loaded TGF- β 1. The concept of enriching the defect site with GFs from the synovial fluid, by injecting a heparan sulfate based gel in a rabbit osteochondral defect model, has also been described and reported good results [76].

The second part of the study consists of a TGF- β 1 dose screening. Of note, the effect of GFs depends on the cell type used [39]. FGF-2 and IGF-1 did not stimulate extracellular matrix deposition by hCCs, when supplemented in the culture medium at concentrations commonly used in the literature (Fig. C1). TGF- β 3 was used in a previous study investigating the potential of the combination of hCCs and HA-TG [64]. TGF- β 3 had been shown to enhance neocartilage formation by chondrocytes *in vivo* when bound to heparin, but was nevertheless also reported not to bind to heparin [53]. We confirmed that TGF- β 3 did not bind to the heparin used in our system (Fig. C2) and focused on the heparin-binding isoform TGF- β 1. The screening also highlighted a dose response with a threshold at 5 ng/ scaffold (equivalent to 200 ng/ml) to promote chondrogenesis to similar extent as TGF- β 1 medium supplementation, despite the 9-fold reduction of the total amount of GF administered to the cells (considering 10 ng for each medium change, 3 times a week for 3 weeks). Of note, the dose of 200 ng/ml is close to the reported amount of 300 ng/ml of TGF- β 1 present in the native cartilage tissue, in its latent form [78]. This amount is low compared to loaded TGF- β doses reported in the literature, such as 50 ng per 4.5-mm diameter, bilayered aginate-PLGA scaffolds [79], 2.5 to 5 μ g/ml in fibrin scaffolds [80], 3 μ g/ml in a PEG hydrogel [81]. The size of defects treated by ACI can reach 10 cm² [83], which would represent, considering a thickness of 2 mm and the use of a scaffold loaded with 200 ng/ml of TGF- β 1, the injection of a total as low as 400 ng of TGF- β 1. However, we also report increased collagen X deposition for all doses of TGF- β 1 that were found to stimulate matrix synthesis.. This can be expected considering that exposure to a high amount of TGF- β 1 can lead to a shift from Smad2/3 to Smad1/5 signaling pathway [85], this latter being

involved in chondrocyte hypertrophy [86]. We can hypothesize that the reduced gene expression levels of Collagen type X at day 21, which seemingly contradicts histological observations, may be linked to the decreasing concentration of TGF- β 1 overtime, which suggests that the collagen X deposition phase could be transient. Of note, the production of collagen type I is part of the normal process of cartilage formation [71]. Since fetal cartilage tissue features were observed after 21 days in culture in this study, a longer time point to observe potential ECM remodeling and maturation and change of composition would give more information about whether our system mimics developmental engineering or promotes the deposition of a fibrocartilaginous ECM.

Lastly, a brief exposure to a high dose of TGF- β 1 has been reported to enhance chondrogenesis *in vitro* [25, 67, 82, 87, 88]. However, studies focusing on the duration of TGF- β administration for stem cell chondrogenesis use various set ups leading to contradicting conclusions, for instance exposure to 5 ng/ml of TGF- β 1 during 2D expansion and pellet assay of human MSCs [88] or 10 ng/ml after encapsulation of expanded human MSCs in a PEG-based scaffold [67] or also bovine and equine MSCs expanded with FGF2 and exposed to 10 ng/ml TGF- β 1 after encapsulation in a self-assembling peptide hydrogel [82]. We compared the efficiency of a burst vs sustained release, using the second highest dose of TGF- β 1 tested in our dose screening, which led to the highest proliferation, ECM deposition and stiffness (20 ng/scaffold, i.e. 800 ng/ml). We demonstrated that, in the system described in this present study, the sustained release of TGF- β 1 was necessary for proliferation of hCCs and matrix production. As such, we consider HA-TG/Hep-TG to pursue the development of an injectable, GF-loaded scaffold for cartilage repair.

5. Conclusion

We have shown that covalent addition of heparin to the HA-TG system allows to sustain the release of TGF- β 1 from HA-TG without affecting its ability to be injectable, biocompatibility or the chondrogenic potential of the cells. We have successfully used this hydrogel as a tool to screen a range of TGF- β 1 concentrations and determined an efficient dose of loaded TGF- β 1 between 200 and 800 ng/ml, and showed that the slow release is necessary to stimulate matrix

deposition by hCCs. This paves the way to an *in situ* crosslinking, minimally-invasive therapy for cartilage lesions, with increased safety due to the low, sustainably released loaded dose of TGF- β 1.

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Author contributions

Conception and design: C.L, M.Z.W, Acquisition and analysis of the data: C.L, Interpretation of the data: C.L, M.Z.W, Drafting of the article: C.L, Critical revision for important intellectual content: M.Z.W, L.A.A, Final approval of the article: M.Z.W, Provision of study materials and patients' samples: L.A.A. provided the human chondroprogenitor cells

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Competing interest statement

None

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Fig. 1: Schematic of the study design. (A) Acellular constructs were used for the characterization of the gelation behavior with rheology, as well as the release kinetics of heparin and of the initially-loaded TGF- β 1 (transforming growth factor β 1) after gelation. (B) Human chondroprogenitor cells (hCCs) were encapsulated in either HA-TG, HA-TG with covalently bound heparin (HA-TG/Hep-TG) or HA-TG with non-covalently bound heparin (HA-TG/Hep). A comparison of the two different heparin incorporation methods was conveyed in terms of extracellular matrix production after 21 days. Further experiments comparing TGF- β 1 administration method (loaded in the gel vs medium-supplemented) did not include HA-TG/Hep. Chondrogenesis was evaluated by characterizing matrix deposition via histology and biochemical assays, measuring chondrogenic genes expression with RT-qPCR as well as quantifying the stiffness with an unconfined compression test.

Fig. 2: Only covalent addition of heparin both efficiently retains TGF- β 1 and promotes cartilaginous matrix deposition. (A) Gelation properties. Gelling curves obtained with a rheometer showed the storage modulus (unbroken lines) and the loss modulus (dotted lines) for two concentrations of HA-TG (2% in light blue and 1% in grey) and 2 heparin incorporation strategies (unbound in pink and crosslinked in dark blue). N=3 for each condition. **(B) Heparin release.** Release curves of heparin (pink, n=3) and Hep-TG (blue, n=4) from the gels over 2 weeks measured with a DMMB assay. Student t test analysis for the last timepoint. **(C) TGF- β 1 release.** Cumulative release curve of TGF- β 1 from HA-TG/Hep gels (pink, n=3), HA-TG/Hep-TG gels (dark blue, n=3) and HA-TG gels (light blue, n=3) over 2 weeks. Student t test analysis for the last timepoint. **(D) ECM deposition by encapsulated hCCs.** GAGs and collagen type II production by hCCs encapsulated in either HA-TG with TGF- β 1 supplemented in the medium (10 ng/ml), HA-TG/Hep or HA-TG/Hep-TG with TGF- β 1 loaded in the gel at 800 ng/ml (20 ng/scaffold), n=4 for each condition. Scale bars = 200 μ m (close-up) and 1 mm (inset). Histological controls are provided in Fig. B.

Fig. 3: hCCs chondrogenic potential is not affected upon covalent addition of heparin in HA-TG, when cultured in TGF- β 1-supplemented medium. **(A) Schematic** **(B) Cellular viability and proliferation** assessed with (i) Live Dead staining, scale bar: 100 μ m and (ii) Picogreen assay respectively. **(C) GAG deposition.** (i) Safranin O staining (n=3), (ii) DMMB quantification in papain-digested gels (n=4) and (iii) gene expression of aggrecan at d21 (n=3). **(D) Collagen type I and II deposition.** (i) Immunohistochemical stainings (n=3) and (ii) gene expression at d21 (n=3). **(E) Mechanical characterization.** Compression modulus after 21 days (kPa), n=4. For all histology images, scale bar: 200 μ m (close up) and 1 mm (insets).

Fig. 4: Initially loaded TGF- β 1 supports hCCs viability and proliferation to a similar level as when continuously medium-supplemented, from a dose of 5 ng/scaffold **(A) Schematic (B) Viability quantification**. Live dead images quantified with ImageJ (n=3). "M" condition: 10 ng/ml TGF- β 1 supplemented in medium). **(C) Proliferation**. Picogreen assay, n=4. **** represents $p < 0.0001$. **(D) Viability assay**. Live dead staining of scaffolds after 3 weeks of culture in serum-free medium. n=3. Scale bars: 100 μ m.

Fig. 5: Initially loaded TGF- β 1 supports homogeneous matrix deposition from a dose of 5 ng/scaffold with a positive dose response **(A) Collagens deposition**. Immunohistochemical stainings of Collagen type II, I and X. Scale bars: 200 μ m (close up) and 1 mm (inserts). N=3. **(B-D) Collagens gene expression** as fold increase compared to day 0 for (B) Collagen type II, (C) Collagen type I, (D) Collagen type X. "M" For medium supplementation. N=3 for each condition. **(E) Stiffness**. E moduli (also referred to as compressive moduli) obtained with an unconfined compression test, n=4. **(F-H) GAGs deposition**. Safranin O staining for (F) loading conditions (dose / scaffold indicated on the top left of the insets) and (G) controls. Scale bars: 200 μ m (close up) and 1 mm (inserts). N=3. (H) GAGs quantification with DMMB assay on papain-digested gels, normalized to the background measured at day 0. N=4 for each condition.

Fig. 6: Sustained release of TGF- β 1 is required for matrix deposition in vitro. Importance of the sustained release for chondrogenesis. **(A) Schematics.** **(B) Viability, Proliferation and Mechanical properties.** (i) Representative Live-dead staining images (scale bar: 100 μ m) and (ii) plot of quantified values (n=3).

(iii) Proliferation data obtained with a Picogreen assay, n=4. (iv) Elastic (E) modulus measured with an unconfined compression test, n=4. **(C) GAGs deposition.** (i) Representative images of Safranin O stainings (n=3) and (ii) plotted values of quantified amounts of GAGs deposited, either normalized to the background at d0 (left) or to the amount of DNA (right), n=4. (iii) Plotted value of Aggrecan gene expression fold increase compared to d0. **(D) Collagens deposition.** Collagen type I and II representative immunohistological stainings (i) and plotted relative amount of gene expression relative to d0 (ii). For all histological images, scale bar: 200 μ m (close up) and 1 mm (inserts).

Supplementary figures

Fig. A: NMR confirms the presence of vinylsulfone peaks and QUANTI-blue assay validates the absence of endotoxins from the polymer solutions **(1) Validation of two major steps of Hep-TG synthesis.** Proton-NMR of the starting heparin (blue), the intermediate vinylsulfonated heparin (green) and the final heparin-TG (red). The location of the peaks corresponding to the vinylsulfone moieties is squared in the 3 stacked spectra and pointed out in the middle spectrum with an arrow. **(2) Validation of the absence of endotoxin in starting / final products.** Material from animal origin bear the risk of containing endotoxins, whose amounts are regulated by ISO guidelines. Quantification of endotoxin performed with a QUANTI-Blue assay. Standard curve (top) and measured values for starting and final products (bottom) are shown as separate graphs, as most of the values found were under the detection limit. Of note, the concentrations were chosen based on the concentration to which the cells are exposed to, but lowered to an amount that is not leading to a viscous solution for the HA. The limit of 0.57 nm of absorbance corresponds to an endotoxin concentration of 0.05 ng/ml (or 0.5 EU/ml considering the acknowledged conversion of 10 EU/ml = 1 ng/ml), which is the limit fixed by the FDA for medical devices.

Fig. B: Histological controls **(1) Day 0 background**. Gels made on the day of the experiment were directly fixed and processed for paraffin embedding (due to the high water content of the gels and the dehydration steps of paraffin embedding, an important shrinkage can be observed, leading to potential increase of staining intensity due to the wrinkling). Safranin O seems to have an increased intensity in HA-TG/Hep-TG compared to HA-TG, despite the low amount present in the gel (0.1%) and although this increased intensity is visible to a lower extent in HA-TG/Hep. Collagen type II staining is positive in the cells but not in the scaffold. **(2) Day 21 background for immunohistochemistry (IHC)**. Representative images obtained in absence of primary antibody (the rest of the protocol being identical).

Fig. C: IGF1, FGF2 and TGF- β 3 fail to either promote ECM deposition when supplemented in the cell culture media or bind to bind to heparin in HA-TG/Hep-TG **(1) Preliminary screen of different GFs widely used for cartilage tissue engineering.** Safranin O staining of 5 μ m-thick cryosections (samples were soaked in 10% sucrose for 1 hour and 30% sucrose overnight, infiltrated at room temperature in O.C.T (Tissue-Tek) and snapfrozen, then cut at a cryostat CryoStar NX70 (Thermo Scientific). Scale bars: 100 μ m (close up) and 500 μ m (inserts). N=2. **(2) Release kinetics of TGF- β 3**, from HA-TG (light blue) and HA-TG/Hep-TG (dark blue). **(3) Release kinetics of TGF- β 1**, from HA-TG (light blue) and HA-TG/Hep-TG (dark blue). For (2) and (3), the curves represent the cumulative percentage of the initially loaded amount of TGF- β , released in the supernatant.

Fig. D: TGF- β 1 burst release does not promote chondrogenesis at any of the tested doses **(1) Viability**. Plot of Live dead images quantification with ImageJ (n=3). **(2) Proliferation**. Picogreen assay, n=4. **(3-6) Collagens gene expression** as fold increase compared to day 0 for (3) Collagen type II, (4) Collagen type I, (5) Collagen type X and (6) Aggrecan. N=3 for each condition. **(7) Quantification of GAGs in scaffolds with DMMB assay**. N=4 for each condition. **(8) Mechanical properties**. E modulus measured with an unconfined compression test, n=4. For all plots, "M" indicates the condition with TGF medium supplementation.