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Bioactive decellularized cardiac extracellular matrix-based hydrogel as a sustained-release platform for human adipose tissue-derived stromal cell-secreted factors

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Abstract

The administration of trophic factors (TFs) released by mesenchymal stromal cells (MSCs) as therapy for cardiovascular diseases requires a delivery vehicle capable of binding and releasing the TF in a sustained manner. We hypothesized that hydrogels derived from cardiac decellularized extracellular matrix (cardiac dECM) bind MSC secretome-derived TF and release these in a sustained fashion. Pig-derived ventricular tissue was decellularized, milled to powder, digested, and assembled as a hydrogel upon warming at 37 °C. The conditioned medium (CMed) of adipose tissue-derived stromal cells (ASC) was collected, concentrated, and incorporated into the hydrogel at 1×, 10×, and 100× the original concentration. The release of 11 ASC-secreted factors (angiopoietin-1, angiopoietin-2, fibroblast growth factor-1, hepatocyte growth factor, platelet-derived growth factor-AA, vascular endothelial growth factor, interleukin-1β, interleukin-6, interleukin-8, CCL2, and matrix metalloproteinase-1) from hydrogels was immune assessed. Bioactivity was determined by endothelial cell proliferation, function, and assessment of endothelial mesenchymal transition. We showed that dECM hydrogels could be loaded with human ASC-secreted TFs, which are released in a sustained manner for several days subsequently. Different trophic factors had different release kinetics, which correlates with the initial concentration of CMed in the hydrogel. We observed that the more concentrated was the hydrogel, the more inflammation-related cytokines, and the less pro-regenerative TFs were released. Finally, we showed that the factors secreted by the hydrogel are biologically active as these influence cell behavior. The use of dECM hydrogels as a platform to bind and release paracrine factors secreted by (mesenchymal) cells is a potential alternative in the context of cardiovascular regeneration.

1. Introduction

The use of stem cells to treat heart failure has been under investigation for two decades. Besides constructive stem cells that differentiate directly to cardiomyocytes or endothelial cells, reconstruction of the damaged myocardium may prevent and reverse disease progression. Heart failure is both the result of a deteriorated pump function along with an exaggerated response of the connective tissue in the form of excessive extracellular matrix deposition [1–3]. We argued that the derailed myocardial tissue requires to be re-educated. In normal physiology, tissue homeostasis is maintained by the tissue stroma, i.e. extracellular matrix, mesenchymal cells and vasculature. As it appears, cultured stromal cells from bone marrow (BM-MSC) or adipose tissue (ASC) are potent tissue remodelers via secretion of a plethora of paracrine factors that suppress apoptosis, promote the proliferation of parenchyma, modulate immune reactions

and are pro-angiogenic [4–6]. These mesenchymal stromal cells (MSCs), including ASC, also remodel extracellular matrix through their secretion of, e.g. matrix metalloproteinases (MMPs) and deposition of extracellular matrix (ECM) components. These MSC are widely used in cardiac cell-based therapies, albeit their mode of action has become (partially) clear only in recent years. The ASC secretome comprises angiogenic factors, immunomodulatory factors, mitogenic factors and ECM-remodeling proteases, among others. That means MSC, by secreting trophic factors (TFs), may augment repair of cardiac tissue damage and to reinstate homeostasis [7, 8]. These factors may directly normalize the function of already present cells or instruct surrounding cells to facilitate repairs. Two significant mechanisms that underlie cardiac fibrosis and thus heart failure and which are influenced by TF are endothelial mesenchymal transition (EndMT) and myofibroblast differentiation of cardiac fibroblasts [9, 10].

Clinical cardiac stem cell therapy has proven of marginal benefit to treating myocardial infarction and cardiac fibrosis [11, 12]. The main reason is the poor retention of stem cells, irrespective of the route of administration. Thus, it is not only essential to warrant the retention of therapeutic cells in the cardiac tissue, but also to ensure that their secreted TF reaches the affected cardiac target cells to direct tissue regeneration. Instead of administration of the TF-secreting MSCs, the administration of the TF themselves is an alternative that reduces possible donor- and disease-dependent variations in TF composition. The challenge, however, is to enable the sustained release of injected TF because usually these are rapidly drained from damaged tissue. To accomplish this task, it is necessary to use a delivery vehicle capable of binding the TF and releasing these in a sustained manner. Several synthetic hydrogels with different tunable characteristics have been proposed as injectable release vehicles to perform this task. In general, these platforms show a burst-release kinetic profile while they efficiently bind all factors in the MSCs' secretome. Recently, our group demonstrated the use of natural hydrogels based on decellularized extracellular matrix (dECM) of adipose tissue as a release platform for secreted paracrine factors in the context of skin wound regeneration [13], corroborating the hypothesis that proteoglycans and glycosaminoglycans (GAG), but also non-fibrillar proteins and matricellular proteins in the ECM, bind the TF present in the cellular environment [14]. *In vivo*, the ECM functions to store and on-demand release these factors physiologically [15, 16]. Tissue-derived ECM hydrogels are promising novel moiety to treat myocardial disease because these mold to the shape of the damaged organ in combination with a sustained release of therapeutic factors [14, 15, 17].

In this study, we hypothesized that hydrogels derived from decellularized cardiac tissue bind MSC secretome-derived TF and release these in a sustained fashion. We investigated the feasibility of fabricating hydrogels derived from porcine myocardial ECM and evaluated the uptake and release of human ASC-derived paracrine factors by these hydrogels. Finally, we explored the influence of the TF released by the hydrogels on endothelial cell proliferation, function, and EndMT.

2. Methods

2.1. Ethical statement

This study was carried out in accordance with the principles outlined in IOP Publishing ethical policy. Human fat tissue, considered waste material, was collected after patients' informed consent and their use was approved for research purposes by the Institutional Review Board of the University Medical Center Groningen, the Netherlands. The study was judged as not falling under the scope of the Medical-Scientific Act for research with humans (METc 2015.584).

2.2. Study overview

An overview of the methods used in the present study is illustrated in figure 1. The detailed information for each method is described below.

2.3. Extracellular matrix decellularization, characterization and dECM hydrogels fabrication

2.3.1. Decellularization protocol

Porcine hearts (16 week pigs) were acquired through a local slaughterhouse (Kroon Vlees, Groningen, Netherlands) and dissected to separate the left ventricle myocardial tissue. Tissue was washed in phosphate buffer saline (PBS) and triturated in a commercial blender until tissue fragments were smaller than 1 mm. Following the 2nd wash in PBS, tissue was submitted to sonication for 1 min, washed a 3rd time, and incubated in 0.05% trypsin in PBS (Gibco, Thermo Fisher Scientific, Waltham, USA) and constant shaking at 37 °C for 3 h. After trypsin treatment, tissue was rewashed with PBS and frozen overnight or longer at −20 °C. After thawing, tissue was incubated in demineralized water for 3 h and then in saturated (6 M) NaCl for another 3 h, both steps in constant shaking. Next, tissue was washed in 70% ethanol for 10 min and in water for another 10 min. After ethanol wash, tissue was collected, centrifuged at 3000 $\times g$ for 3 min, discarded the supernatant followed by tissue resuspension and incubation with 1% sodium dodecyl sulfate (SDS) in water (Sigma-Aldrich, St. Louis, USA) for 12 h, washed three times with water, incubated with 1% Triton X-100 (Sigma-Aldrich, St. Louis, USA) for 12 h, washed three times with water, incubated with 1% sodium deoxycholate (Sigma-Aldrich, St. Louis, USA) for 12 h and, again, washed three times with water, all steps under constant shaking. After detergent treatment and washing, tissue was incubated for 24 h with DNAse solution

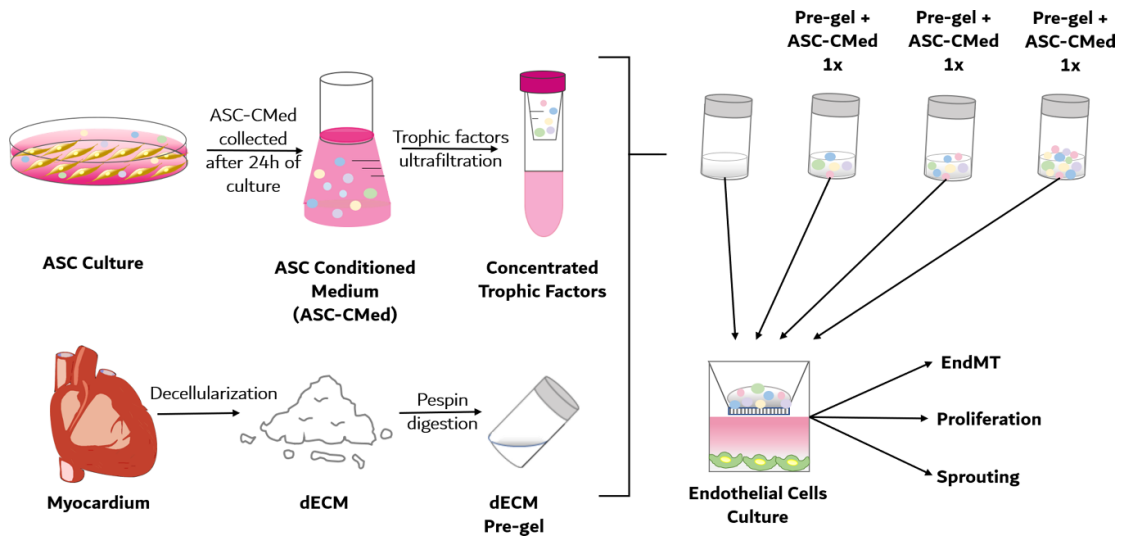

Figure 1. Overview of the study design.

(30 µg ml^{-1} DNAse (Worthington Biochemical Corporation, Lakewood, USA), 1.3 mM $MgSO_4$, 2 mM $CaCl_2$) at 37 °C and constant shaking. After DNAse treatment, tissue was washed overnight with 70% ethanol and stored at 4 °C in 1% penicillin/streptomycin (#15 140 122, Gibco Invitrogen, Carlsbad, USA) in sterile PBS. In the following the final product is denoted as dECM.

2.3.2. DNA quantification

Genomic DNA was isolated as previously described [13]. Briefly, dried native and dECM samples ($n = 3$) were weighed and separated as samples of 10–15 mg in a 1.5 ml Eppendorf tube. Each sample was digested at 55 °C overnight in a solution containing 162 µg ml^{-1} proteinase K, 10 mg ml^{-1} SDS, and 500 µl SE-buffer (75 mM NaCl; 25 mM EDTA; pH 8.0). Following the enzymatic digestion, samples were added with 222 µl of 6 M NaCl and 777 µl of chloroform, thoroughly shaken on a top-over-top rotator for 1 in room temperature (RT) and centrifuged at 2000×*g* at 20 °C for 10 min. After centrifuging, two liquid layers were formed with a white layer in between. The upper layer of the supernatant was pipetted out of the original tube and transferred into a new clean one, leaving the protein pellet behind. In the new tube, a volume of ice-cold isopropanol was added to the transferred supernatant and gently mixed until white threads of DNA formed visible clumps. The tubes were then centrifuged at 12 000×*g* at 4 °C for 15 min, forming a pellet. The supernatant was carefully pipetted out, without disturbing the DNA pellet, and 500 µl of 70% ethanol was added to wash the pellet. The tubes were centrifuged again at 12 000×*g* at 4 °C for 5 min, the supernatant was pipetted out, and the pellet was left to air-dry at RT. Once the pellet was dissolved in 100 µl of TE-buffer (10 mM Tris; 0.1 mM EDTA; pH 8.0) at 55 °C and quantified with NanoDrop spectrophotometry (Thermo Scientific, Hemel Hempstead, United Kingdom).

2.3.3. dECM gelation

For dECM gelation, the decellularized extracellular matrix of left ventricle myocardial tissue in PBS was centrifuged at 12 000×*g* at 4 °C for 5 min. The supernatant was discarded and the tissue pellet lyophilized and milled to a fine powder. Digestion was done on 20% w/v dECM powder in 0.01 M HCl with 2% w/v pepsin (#P6887; >3200 IU; Sigma-Aldrich, St. Louis, USA) at RT under constant stirring for 6 . After digestion, the pH was neutralized by adding 1/10 volume 0.1 M NaOH and brought to 1× PBS by adding 1/10 volume 10× PBS. The resulting solution could be stored in liquid form (pre-gel) at 4 °C for months or converted into a hydrogel by warming to 37 °C, which induced spontaneous gelation. The hydrogel characterization was performed as described in previous work from our group [13, 14, 18].

2.4. Grow factors from ASC-CMed released by dECM hydrogels

2.4.1. Conditioned medium collection, concentration and resuspension in dECM hydrogel

Human ASC were isolated and characterized as described previously [9, 19, 20]. Briefly, human abdominal fat was obtained by liposuction, washed with PBS and digested enzymatically with 0.1% collagenase A (#11088793001, Roche Diagnostic, Mannheim, Germany) in PBS with 1% bovine serum albumin (BSA; #A9647, Sigma-Aldrich, Boston, USA). The tissue was shaken constantly at 37 °C for 2 h. After this, the digested tissue was mixed with 1% PBS/BSA, filtered, centrifuged and the cell pellet was resuspended in Dulbecco's Modified Eagle's

Medium (DMEM; #12-604F, Lonza, Basel, Switzerland) with 10% fetal bovine serum (FBS; #F0804, Sigma-Aldrich, Missouri, United States), 1% penicillin/streptomycin (#15140122, Gibco Invitrogen, Carlsbad, USA) and 1% L-glutamine (#17-605E, Lonza Bio Whittaker, Verviers, Belgium). Cells were cultured at 37 °C in a humidified incubator with 5% CO_2. The medium was refreshed every 2 d. Cells were passed at a ratio of 1:3 after confluency had reached.

ASC conditioned medium (ASC-CMed) was obtained from confluent cultures of ASC between passages 3 and 6 from at least three different donors. Cells were cultured in DMEM, and the serum-free CMed (5 ml cm^{-2}) was harvested after 48 h, filtered through 0.22 μm filters. ASC-CMed was concentrated with the use of 3 kDa cutoff Amicon® Ultra Centrifugal filters (Sigma-Aldrich). ASC-CMed was concentrated 2000-fold by repeating the concentrating procedure with previously concentrated samples. After concentration, ASC-CMed was diluted in DMEM to reach the following concentrations: 2000×, 200×, and 20×. These ASC-CMed solutions were then added as 1/20 final volume to dECM pre-gel, resulting in pre-gels containing, respectively, 100×, 10×, and 1× ASC-CMed.

2.4.2. Trophic factors release from dECM hydrogel

ASC-CMed-containing dECM pre-gels were fabricated as described above. In sequence, 500 μl of each pre-gel (1× ASC-CMed, 10× ASC-CMed, and 100× ASC-CMed) was added to 1.5 ml microtubes and placed in an incubator for 1 h at 37 °C to allow for self-assembly gelation. This yields 2% w/v dECM hydrogels. After gelation, 500 μl of serum-free DMEM, without any additional factors, was added to the dECM hydrogel, and the microtubes were kept closed inside a 37 °C incubator. The supernatant was collected from the microtubes and refreshed at 1, 3, and 5 d, constituting, respectively, the 1st, 2nd, and 3rd waves of TF release. The collected supernatant was used to determine trophic factors released from dECM hydrogel over time.

The factor release profile of dECM hydrogel was determined by the concentration of 11 factors that are representative for tissue regeneration and remodeling (angiopoietin-1, angiopoietin-2, fibroblast growth factor 1 (FGF-1), hepatocyte growth factor (HGF), platelet-derived growth factor-AA (PDGF-AA), vascular endothelial growth factor (VEGF), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemotactic protein 1 (CCL2/MCP-1), and matrix metalloproteinase 1 (MMP-1)) in the medium collected from the dECM hydrogel. For this purpose, Magnetic Luminex Human Premixed Multi-Analyte Kit (R&D Systems) was used according to the manufacturer's protocol. Serum-free DMEM only and DMEM extracted from dECM hydrogels without ASC-CMed were used as negative controls, while the concentrated ASC-CMed was used as a positive control.

2.5. Endothelial cells' proliferation, function, and transdifferentiation under ASC-CMed release by dECM hydrogels

2.5.1. Cell sources and cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from the endothelial cell culture facility of our institution and comprised pools of at least three donors, as previously described [10]. Cells were seeded on gelatin-coated plates (1% gelatin solution in PBS) at a density of 35 000 cells cm^{-2} and cultured until confluency in endothelial cell medium (ECMed) composed of RPMI-1640 basal medium (#BE04-558F, Lonza, Basel, Switzerland) with 10% heat-inactivated FBS (#F0804, Sigma-Aldrich, Missouri, United States), 1% penicillin/streptomycin (#15140122, Gibco Invitrogen, Carlsbad, USA), 1% L-glutamine (#17-605E, Lonza Bio Whittaker, Verviers, Belgium), 5 U ml^{-1} heparin (LEO Laboratories Limited, Ballerup, Denmark), and 50 g ml^{-1} bovine brain extract (BBE, in-house preparation). HUVEC between passages 3 and 6 were used for the experiments.

2.5.2. Endothelial cells proliferation

A transwell membrane insert ThinCert™ with 0.4 μm (#662640, Greiner Bio-One International, Kremsmünster, Austria) was used with hydrogel and/or the concentrated CMed on top of 24 well tissue culture plates of HUVEC. After 5 d of culture with ECMed with 10% FBS and endothelial cell growth factor (ECGF), cells were fixed at RT with 2% paraformaldehyde (PFA) for 30 min and stained as described below (see Immunofluorescence section)

2.5.3. Endotshelial cell function

ASC-CMed-containing dECM pre-gels were generated as described above. In sequence, 500 μl of each pre-gel (without ASC-CMed, 1× ASC-CMed, 10× ASC-CMed, and 100× ASC-CMed) was added to 1.5 ml microtubes and placed in an incubator for 1 h at 37 °C to allow for self-assembly gelation. After gelation, 500 μl of ECMed, without any additional factors, was added to the dECM hydrogel, and the microtubes were kept closed inside a 37 °C incubator for 24 h. After this time, the 500 μl of ECMed were pipetted out from the microtubes and stored for use in the endothelial sprouting assay.

HUVEC were cultured with ECMed in 75 cm^2 culture flasks. After reaching confluency, cells were detached from the flasks, counted, and, for each group, 15 000 cells were resuspended in 50 μl of the ECMed collected from the microtubes as described above. Pure ECMed was used as a positive control. Subsequently, cells were seeded in wells of a μ-Slide Angiogenesis Plate (Ibidi GmbH, Martinsried, Germany) previously coated and incubated at 37 °C with

Table 1. Experimental groups.

Group	EndMT	Hydrogel	Secretome
Control	—	—	—
Control + Induction	+	—	—
Hydrogel	—	+	—
Hydrogel + Induction	+	+	—
Hydrogel with 1× ASC-CMed	—	+	1×
Hydrogel with 1× ASC-CMed + Induction	+	+	1×
Hydrogel with 10× ASC-CMed	—	+	10×
Hydrogel with 10× ASC-CMed + Induction	+	+	10×
Hydrogel with 100× ASC-CMed	—	+	100×
Hydrogel with 100× ASC-CMed + Induction	+	+	100×

EndMT: Induction of endothelial mesenchymal transition.

10 µl of Matrigel® (#356231,BD Biosciences, San Jose, USA) for 2 h. The sprouting was allowed to proceed for 4 h. Every condition was done in triplicate, and the experiment was performed three times independently. Formation sprouting networks was imaged with a DM2000 LED inverted microscope (Leica, Wetzlar, Germany) using 2.5× magnification and analyzed using ImageJ Fiji with Angiogenesis Analyzer plugin. The number of branches was determined.

2.5.4. Endothelial cell mesenchymal transition (EndMT)

Confluent HUVEC were divided into ten groups with different induction/dECM hydrogel combinations, as described in table 1, and cultured for 5 d. Human recombinant IL-1β (#200-01B, PeproTech, New Jersey, USA) and human transforming growth factor-beta 2 (TGF-β2; #100-35B, PeproTech, New Jersey, USA) both at 10 nl ml^{-1}, were used to induce EndMT.

ASC-CMed-containing dECM pre-gels were generated as described above. In sequence, 500 µl of pregel was added to the inner part of ThinCert™ Cell Culture Inserts 24 Well (Greiner Bio-One) and placed in an incubator for 1 h at 37 °C to allow for gelation. Wells of 24-well plates seeded with HUVEC were allocated within the experimental groups described above. The ThinCert™ Cell Culture Inserts containing dECM hydrogels were then placed above the HUVEC culture according to their experimental groups. The culture medium was refreshed every 2 d, while dECM hydrogel was not changed during the experiment. After 5 d of culture with ECMed with 10% FBS and ECGF, cells were fixed at RT with 2% PFA for 30 min and stained as described below (see section 2.5.5).

2.5.5. Immunofluorescence

After the appropriate period for each experiment, the cells were fixed at RT with 2% PFA in PBS for 30 min. Cells were permeabilized with 1% Triton-X100 in PBS at RT for 15 min and blocked with 5% donkey serum in PBS and 1% BSA at RT for 15 min. Subsequently, cells were incubated with primary antibodies diluted in 5% donkey serum in PBS at RT for 2 h.

For cell proliferation, rabbit anti-Ki67 (1:400; #ab15580, Abcam, Cambridge, UK) polyclonal antibody was used. To visualize EndMT, rabbit anti-SM22α (1:400; #ab14106, Abcam, Cambridge, UK) and mouse anti-human VE-Cadherin (1:200, #AF1002, R&D Systems, Minnesota, United States) were used as mesenchymal and endothelial markers, respectively. Controls were incubated with 5% donkey serum in PBS instead of the primary antibody.

Next, the cells were washed with 0.05% Tween-20 in PBS and incubated with secondary antibodies in 5% donkey serum in PBS with 4′,6-diamidino-2-phenylindole (DAPI; 1:5000; #D9542-5 MG, Sigma-Aldrich, Missouri, United States) at RT for 1 h. For the transdifferentiation experiment, cells were also incubated with Alexa Fluor® 488 phalloidin (1:400; #A12379, Life Technologies, Carlsbad, United States). The following secondary antibodies were used: donkey anti-rabbit IgG (H + L) Alexa Fluor® 594 (1:400; #A-21207, Life Technologies, Carlsbad, United States) and donkey anti-mouse IgG (H + L) Alexa Fluor® 594 (1:400; #ab150108, Abcam, Cambridge, UK). Finally, cells were washed 3 times with PBS and the plates were imaged with Evos FL System (Thermo Fisher Scientific, Waltham, United States) using Texas Red, DAPI and Green Fluorescent Protein channels with 20× magnification.

2.6. Statistical analysis

All data were obtained from at least three independent experiments performed in duplicate or triplicate. Data are presented as mean ± standard error of the mean. Graphs and statistical analyses were done using GraphPad Prism (Version 6.01; GraphPad Software, Inc., La Jolla, United States). Differences among multiple groups were analyzed by Kruskal–Wallis with Dunn's multiple comparison test for the two groups of interest in each scenario.

3. Results

3.1. Effective decellularization

The presence of remaining DNA in the dECM was assessed by genomic DNA quantification. The dry myocardial tissue before decellularization contained

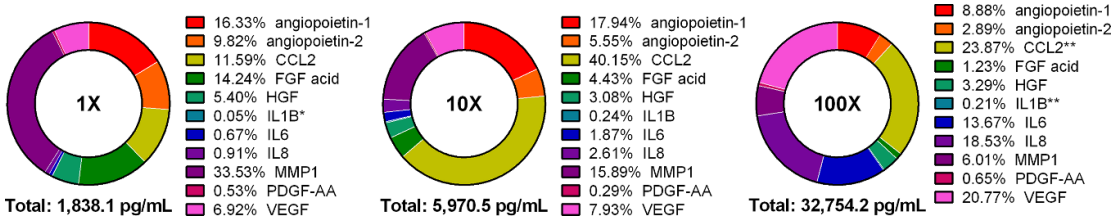

Figure 2. Trophic factor proportion in the collected ASC-CMed (cumulative after 5 d) at different concentrations (1×, 10×, and 100×). *IL-1β was below the detection limit for 1× concentrated hydrogel and **CCL2 was above the detection limit for 100× concentration hydrogel; in these cases the lower and upper thresholds, respectively, were considered for proportion calculation ($n = 4$ independent experiments).

3617 ± 342.1 ng mg^{-1} vs. 14.3 ± 3.6 ng mg^{-1} after the process (t-student test, $p < 0.0001$). Genomic DNA quantification showed around 99% of reduction in DNA content, with dECM presenting less than 50 ng mg^{-1} of DNA per dry weight, the standard value for successful decellularization [21].

The complete characterization of the myocardial dECM hydrogel was previously performed, demonstrating the maintenance of sulfated GAG, structural ECM proteins and also minor ECM-related proteins [14].

3.2. Trophic factor relative concentration varies according to the level of concentration of the CMed

The relative concentration, i.e. the proportion of each released TF, for all the three release waves, in the analyzed medium varied according to the level of concentration of the CMed (figure 2). While in the hydrogels loaded with 1× concentrated CMed the most prevalent released TFs were MMP-1 (616 pg ml^{-1} or 33.53%), angiopoietin-1 (300 pg ml^{-1} or 16.33%), and FGF-1 (261 pg ml^{-1} or 14.24%), for the 10× concentrated CMed they were CCL2 (2397 pg ml^{-1} or 40.15%), angiopoietin-1 (1071 pg ml^{-1} or 17.94%), and MMP-1 (948 pg ml^{-1} or 15.89%). Finally, for the 100× concentrated CMed, the three most prevalent TF were CCL2 (>7820 pg ml^{-1} or >23.87%), VEGF (6804 pg ml^{-1} or 20.77%), and IL-8 (6068 pg ml^{-1} or 18.53%). In general, the more concentrated was the medium, more inflammation-related cytokines and less pro-regenerative TFs were present.

3.3. dECM hydrogels can release trophic factors in a sustained manner, but each factor has its own release kinetics

The TF release profile of dECM hydrogel was determined by the measurement of 11 different factors in the medium collected from the dECM hydrogel after three consecutive waves of release, i.e. a 1st of 24 h and a 2nd and 3rd wave of 48 h, using the Magnetic Luminex Human Premixed Multi-Analyte Kit. Empty control gels did not release detectable amounts of factors (not shown). The release of CCL2 from dECM hydrogels loaded with 100× concentrated ASC-Cmed was above the maximal detection level, while the release of IL-1β from dECM hydrogels loaded with 1× concentrated ASC-CMed was below the detection limit.

Although the increase in ASC-CMed concentration in the hydrogels was exponential (tenfold), this pattern was not observed in the release behavior of individuals by the hydrogels. In general, the hydrogels loaded with 1× and 10× concentrated CMed showed a similar release pattern, with low TF concentration and a relatively constant TF release speed. The 100× concentrated hydrogels, on the other hand, showed different behaviors for each TF (figures 3(A) and (B)), each of them either presenting a sustained release (angiopoitin-1, angiopoitin-2, FGF-acid, and MMP-1) or a strong release in the 1st wave, that leveled off in subsequent waves (PDGF-AA, HGF, VEGF, CCL2, IL6, and IL8).

3.4. Release of regeneration-related trophic factors

Among the 11 TFs investigated in this study, seven potentially augment to tissue regeneration: angiopoietin-1, angiopoietin-2, FGF-1, PDGF-AA, HGF, VEGF, and MMP-1.

The release curve of angiopoietin-1 and angiopoietin-2 showed the same pattern, although the concentration reached by angiopoietin-1 was higher in all time points compared with angiopoietin-2. During the 1st 24 h (1st wave), 100× CMed hydrogels released a mean concentration of 1111 pg ml^{-1} of angiopoietin-1, followed by 542 pg ml^{-1} and 125 pg ml^{-1} for 10× and 1× CMed hydrogels. On the 3rd wave, the mean concentration of this TF was still considerable in all groups, representing at least around 50% of the concentration found in the 1st wave (1st 24 h). For angiopoietin-2, in turn, the mean concentration reached in the 1st wave was 430.3 pg ml^{-1}, 133 pg ml^{-1}, and 64 pg ml^{-1}, respectively, for the hydrogels loaded with 100×, 10× and 1× CMed. After the 3rd wave, a similar behavior to angiopoietin-1 was observed, with a mean concentration of about 50% of the concentration of the 1st wave's release.

FGF-1 and PDGF-AA showed the lowest release among all the regeneration-related TFs investigated in the present study. While for FGF-1 the mean concentrations during the 1st release wave (1st 24 h)

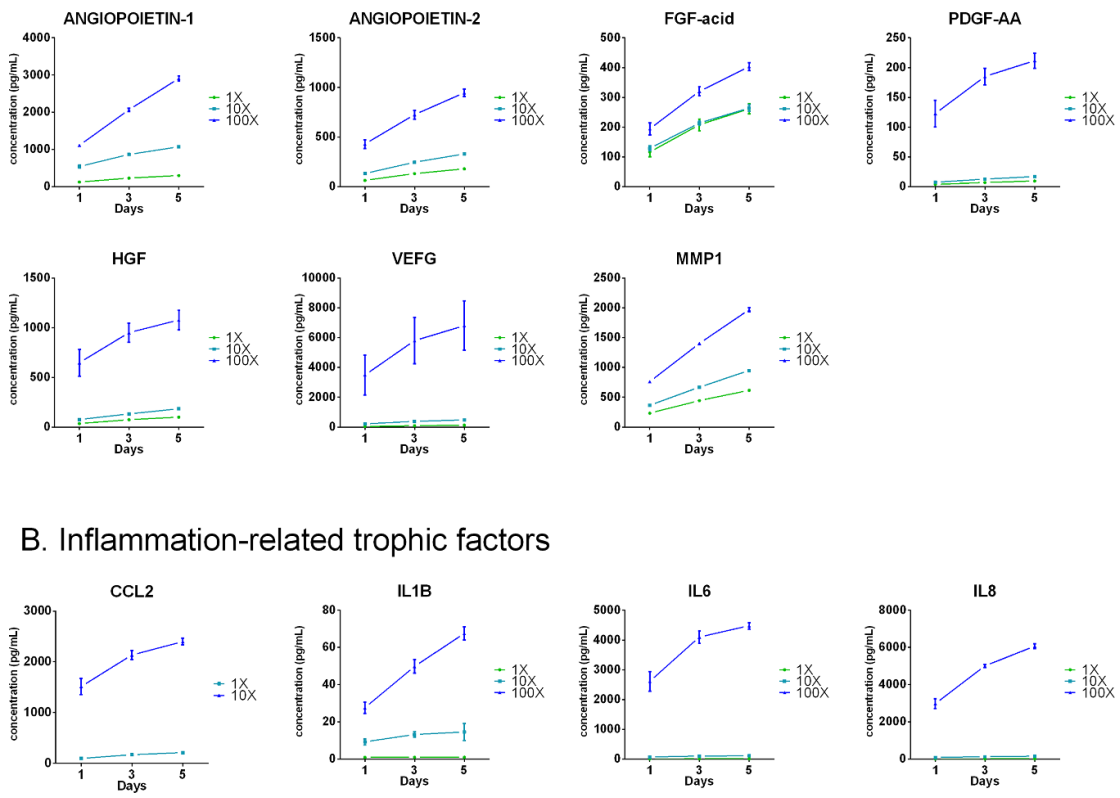

Figure 3. Cumulative concentration of trophic factors over a 5 d release period. (A) Regeneration-related trophic factors release pattern after 1, 3, and 5 d of release. (B) Inflammation-related trophic factors release pattern after 1, 3, and 5 d of release. CCL2 was above the detection limit in 100× concentrated CMed, and IL-1β was below the detection limit in 1× concentrated CMed ($n = 4$ independent experiments).

were 194 pg ml^{-1}, 128 pg ml^{-1} and 116 pg ml^{-1}, respectively for the hydrogels loaded with 100×, 10× and 1× CMed, for PDGF-AA it was 123 pg ml^{-1}, 7.5 pg ml^{-1} and 3.8 pg ml^{-1}. The release kinetics differed between TFs, and there was disparity on concentrations between the most and least concentrated hydrogels, so for FGF-1, hydrogels containing 1× and 100× concentrated CMed did not differ on the released TF amount, while for PDGF-AA, these two concentrations led to a different final load of released TF.

The release of HGF reached a mean concentration in the 1st wave of 647 pg ml^{-1} for the 100× CMed, which decreased fivefold to 127 pg ml^{-1} in the next wave. The 1× and 10× concentrated CMed reached lower maximum concentrations of 35 pg ml^{-1} and 75 pg ml^{-1} in 24 h, and 52 pg ml^{-1} and 25 pg ml^{-1} after the 3rd wave (5 d). VEGF, in turn, showed a more controlled release, with a maximum TF concentration of 3490 pg ml^{-1} for the 100× concentrated CMed at 1st wave, representing the most abundant of all analyzed regeneration-related TFs. Even after 5 d of the experiment, VEGF concentration remained high, with a mean value of 1003 pg ml^{-1}. On the other hand, the 10× and 1× concentrated CMed showed low TF concentrations, 209 pg ml^{-1}, and 40 pg ml^{-1}, respectively, after the 1st wave.

Finally, MMP-1 showed a sustained release along with the experiment. At 1st wave (1st 24 h), the 100× CMed group reached a mean concentration of 761 pg ml^{-1}, which remained stable at 568 pg ml^{-1} at the end of the experiment. The same was true for 10× and 1× concentrated CMed, which reached, respectively, 364 pg ml^{-1} and 232 pg ml^{-1} at 24 h and kept the concentration at 279 pg ml^{-1} and 172 pg ml^{-1}, respectively, by the end of the experiment. Thus, MMP-1 showed a smaller difference between CMed concentrations and the most sustained release curves.

3.5. Release of inflammation-related trophic factors

Among the 11 TFs investigated in this study, four of them were related to inflammation: CCL2, IL-1β, IL-6, and IL-8.

The concentration of released inflammation-related TFs was, in general, higher than that observed for pro-regenerative TFs. CCL2 was, by far, the most released factor. Its concentration was so high that, in the 100× CMed, it surpassed the upper limit of the assay (7820 pg ml^{-1}). Even for the 10× concentrated

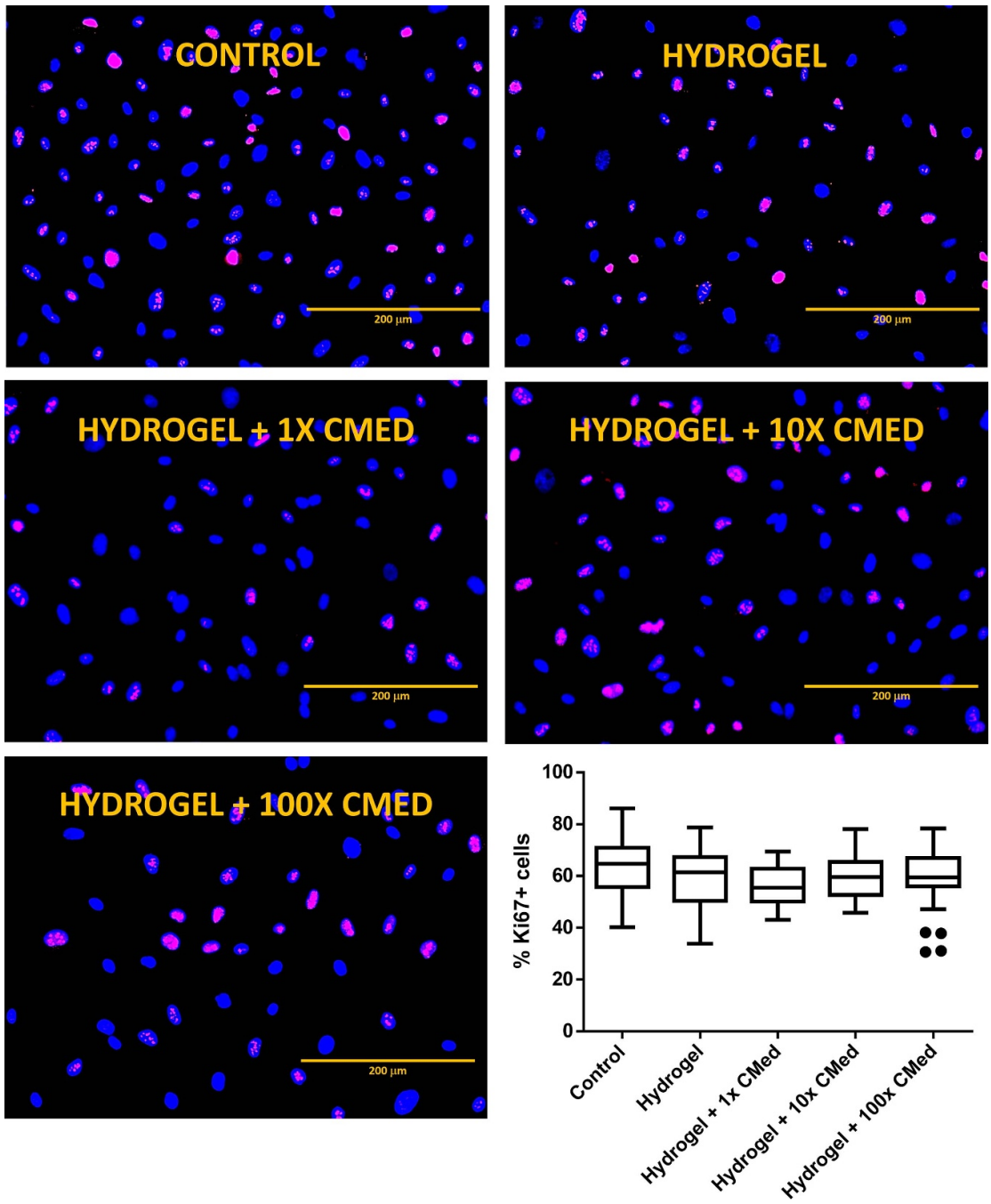

Figure 4. Endothelial cell proliferation after 5 d of co-culture with trophic factors-loaded dECM hydrogel. Blue staining (DAPI) delimitates the nuclei and pinkish staining (Ki-67) is a marker of proliferation. The four dots in the graph represent outliers according to Tukey plotting methodology ($n = 3$ experiments in duplicate).

CMed, the released concentration at 1st wave was high, 1514 pg ml^{-1}, and quickly dropped after that, reaching 263 pg ml^{-1} on the 3rd wave. The 1× concentrated CMed, however, showed lower initial concentrations, 97 pg ml^{-1} in the 1st wave, but a stable release, reaching 43 pg ml^{-1} on the 5th day. In contrast to CCL2, however, was the behavior of IL-1β, for which the minimal concentration was below the lower limit of the assay (2 pg ml^{-1}). Even at 100× concentrated CMed, the cytokine reached no more than 27 pg ml^{-1}, remaining relatively stable at 18 pg ml^{-1} on the 3rd wave. IL-6 and IL-8 showed similar release kinetics, with both cytokines reaching concentrations close to 3000 pg ml^{-1} during the 1st wave for the 100× concentrated CMed (2613 pg ml^{-1} for IL-6 and 2978 pg ml^{-1} for IL-8), but lower than 100 pg ml^{-1} for the 10× and 1× concentrated CMed.

3.6. Influence of the trophic factors released from dECM hydrogels on cell proliferation, endothelial function, and transdifferentiation

The proliferation capacity, endothelial function, and transdifferentiation of HUVEC were accessed after culturing these cells with the TFs

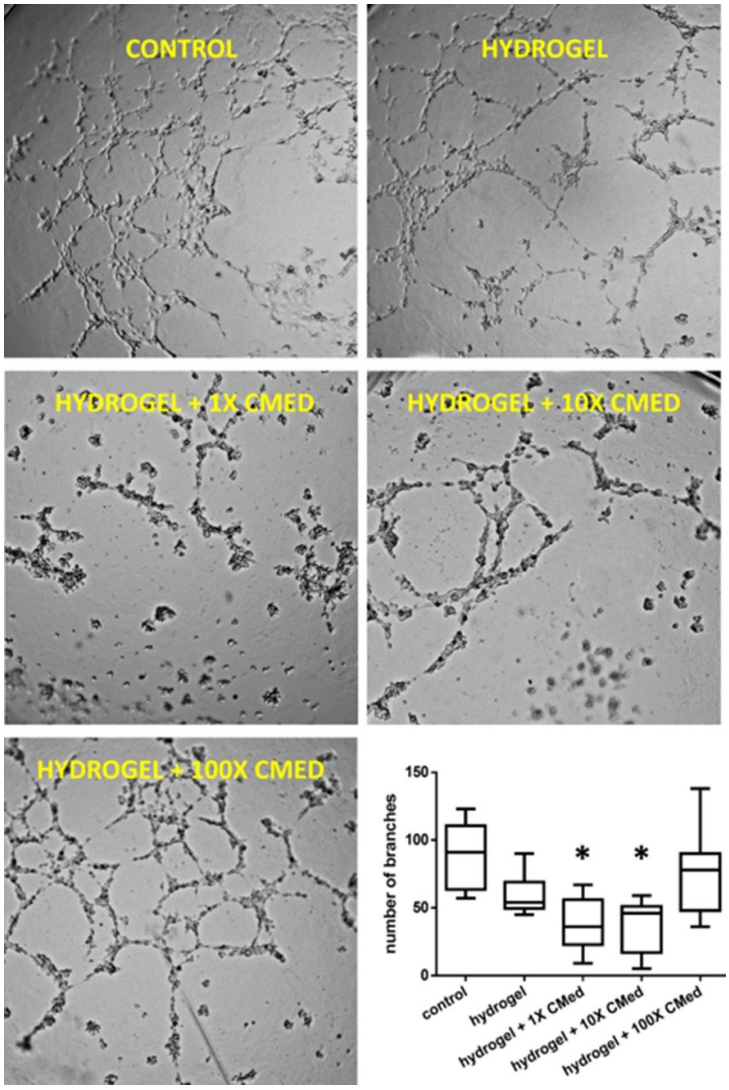

Figure 5. Endothelial cells sprouting after 4 h under the influence of trophic factors released from dECM hydrogels for 24 h. $^{*}p < 0.001$ ($n = 3$ independent experiments in triplicate).

released by the dECM hydrogel loaded with ASC CMed.

Neither hydrogel alone nor the hydrogel loaded with TFs, irrespective of CMed concentration (1×, 10×, and 100×) influenced HUVEC proliferation (figure 4). For all the experimental groups, about 60% of the endothelial cells were proliferative, with no significant differences among them (Kruskal–Wallis, $p = 0.128$).

In contrast to proliferation, endothelial cell sprouting changed markedly upon the application of dECM hydrogels loaded with ASC secretome (figure 5). Factors released from hydrogels loaded with 1× and 10× CMed inhibited *in vitro* endothelial sprouting as observed by a decreased number of branches compared to controls (Kruskal–Wallis, $p = 0.0003$; 1× CMed vs. control, $p = 0.0009$; 10× CMed vs. control, $p = 0.0005$). In contrast, the release of factors from cardiac dECM hydrogels loaded with 100× Cmed did not affect endothelial sprouting. Similarly, bare dECM hydrogel did not affect sprouting.

Finally, we assessed the influence of dECM-released CMed factors on TGF-β-induced EndMT. Controls, i.e. HUVEC without induction, did not show signs of EndMT because mesenchymal marker expression was undetectable (figure 6). Co-incubation of HUVECs with dECM hydrogels in transwells did not induce EndMT, nor did the CMed factors released from these hydrogels (irrespective of their concentration factor). Under these conditions, the endothelial phenotype was maintained as determined by the expression of the endothelial marker VE-Cadherin (figure 6). Induction of EndMT caused the cytoplasmic expression of SM22α (figure 6) in controls and HUVECs exposed to bare dECM hydrogels in transwells. Although there was a trend towards a decreased expression of mesenchymal markers in the experimental groups loaded with 10× CMed, these did not differ from controls (Kruskal–Wallis

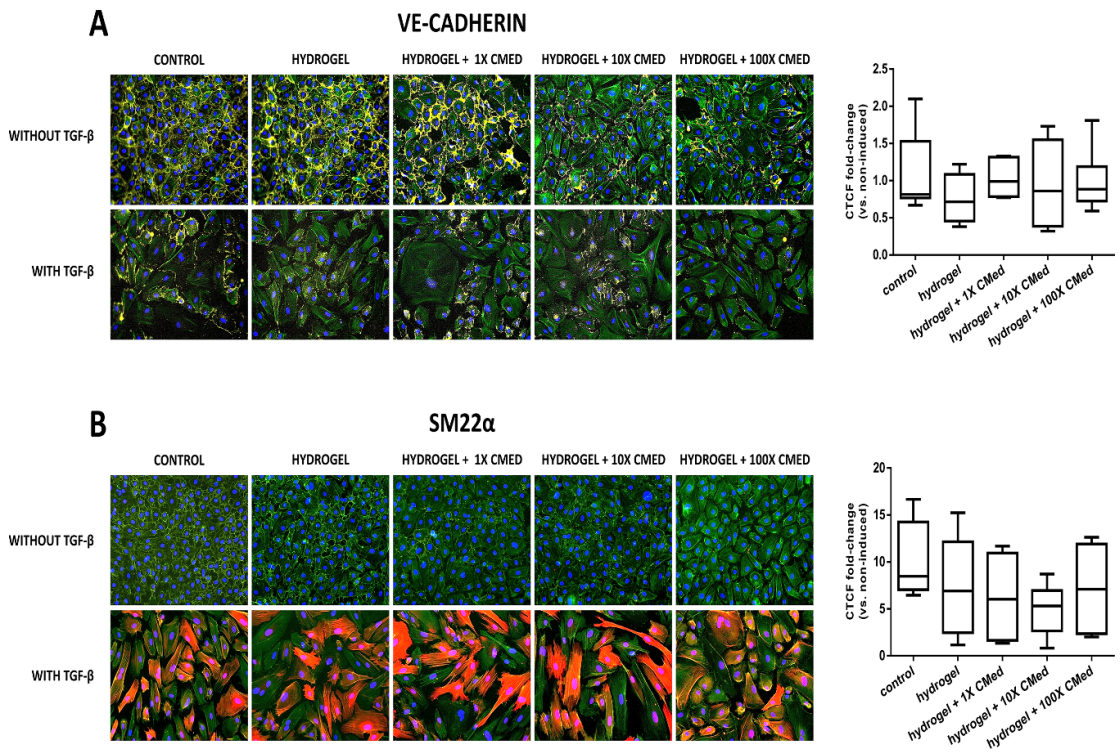

Figure 6. Influence of trophic factors released from dECM hydrogels on endothelial cells transdifferentiation. (A) VECadherin in yellow and phalloidin in green. (B) SM22α in red and phalloidin in green ($n = 3$ independent experiments in duplicates).

$p = 0.284$; 10× CMed vs. control, $p = 0.1220$). Concerning VE-Cadherin expression, EndMT induction reduced VE-Cadherin expression on the endothelial cell membrane. This loss was not rescued by the TFs released from the hydrogel (Kruskal–Wallis $p = 0.653$).

4. Discussion

In the present study, we investigated the use of porcine left ventricle-derived decellularized extracellular matrix hydrogels to release TFs secreted by cultured human adipose tissue-derived stromal cells. We developed a method to produce this TF-loaded hydrogel system and assessed the release pattern of 11 different TFs related to tissue regeneration and assessed their biological activity on endothelial cells. Firstly, we showed that porcine dECM hydrogels could be loaded with human ASC-secreted TFs, which are released in a sustained manner for several days subsequently. Secondly, we demonstrated that these TFs had different release kinetics, which correlates with the initial concentration of CMed in the hydrogel. We observed that the more concentrated was the hydrogel, the more inflammation-related cytokines, and the less pro-regenerative TFs were present. Finally, we showed that the factors secreted by the hydrogel are biologically active as these influence cell behavior, particularly concerning endothelial cell function.

The differential release of the 11 TFs herein investigated points to the possibility that each TF has a different binding saturation threshold. This finding directly affects the outcomes from CMed-based therapies based on the release of TF by hydrogel, particularly dECM hydrogels. We showed that in low concentrations, i.e. hydrogels loaded with 1× CMed, the most prevalent TF released were MMP-1, angiopoietin-1, and FGF-1, all pro-regenerative factors. At this level of CMed concentration, however, the concentration of these factors is also low, probably not enough to produce beneficial results. On hydrogels loaded with 10× CMed, the TF release profile starts to alter towards a more inflammatory-related set of factors, so that, at this concentration, the most prevalent TF is CCL2, already in a considerable concentration (2397 pg ml^{-1}). In fibrotic disease, pro-inflammatory chemokines such as CCL2, orchestrate endothelial cell proliferation and apoptosis resulting in reduced cell repair capacity [22–26] and contributing to EndMT [27–29]. At 100× concentrated hydrogels, the concentration of CCL2 surpassed the upper limit of the assay (7820 pg ml^{-1}), and the two factors that had higher concentrations and were under the upper limit detection of the test were VEGF and IL8. At this concentration, VEGF seems to be the responsible factor for restoring the endothelial cell function, which was lost in hydrogels loaded with 1× and 10× CMed.

The release of TFs has been previously shown to be modulated by several factors, such as GF-scaffold binding, hydrogel material and, in the case of dECM hydrogels, the ECM donor and the presence of diseased matrix, e.g. matrix from diabetic patients [13, 30, 31]. Certainly, one of the main aspects involved in TF release by dECM hydrogels is the binding of such TF to the GAG. These are large, negatively charged polysaccharides that bind both the cells, the other ECM proteins, and the TF. It has been previously shown that even the same TF, in two different isoforms, show variable behavior when binding to GAG [32]. Another factor that can contribute to the release behavior of TF is the presence of MMPs, which cleaves not only ECM proteins but also the binding of these proteins to others [33]. In our study, we investigated only MMP-1 (an interstitial collagenase), but probably other MMPs are also present in the CMed and possibly also expressed in high concentrations, as MMP-1. Thus, the release of the different TFs could be affected by the presence and concentration of MMP.

The use of mesenchymal cells secreted factors, delivered by the dECM hydrogel, was initially proposed, in our study, as a potential approach to inhibit endothelial-mesenchymal transition and maintain endothelial function. Although we sought the opposite effect, with loss of the HUVEC sprouting capacity, we did demonstrate that the factors released by the dECM hydrogels are biologically active, considering the same outcome was not present in the group with dECM hydrogel only. We consider that this negative effect, however, is in accordance with the findings of our LUMINEX assay, which demonstrated a partly inflammatory secretome. Future studies using single or few TFs could better elucidate this issue.

Still, the use of dECM hydrogels as a platform to bind and release paracrine factors secreted by (mesenchymal) cells is a potential alternative to the use of synthetic hydrogels in the context of cardiovascular regeneration. Recently, our group also proposed the use of adipose tissue dECM hydrogels as a release platform to deliver secreted paracrine factors for wound healing applications [13]. To the best of our knowledge, this work is the 1st to demonstrate the use of myocardial dECM hydrogels loaded with TFs derived from mesenchymal cells as a potential approach to cardiac regeneration. The fine-tuning of the manufacturing methods and the appropriate balance between CMed concentration and the release of the right TF remains to be investigated. Herein, however, we demonstrated the feasibility of the method and the differences which can be found when the mesenchymal cells secretome is concentrated on a logarithmic scale. Future studies should focus on how hydrogels from different tissues differ regarding the release of TFs—in different concentrations—but also on the *in vivo* behavior of these hydrogels and the outcomes achieved by dECM hydrogels-based therapies.

Conflict of interest

Authors declare no conflict of interest.

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References

[1] Bui A L, Horwich T B and Fonarow G C 2010 Epidemiology and risk profile of heart failure *Nat. Rev. Cardiol.* **8** 30–41
[2] Benjamin E J *et al* (American Heart Association Statistics Committee and American Heart Association Statistics Committee) 2017 Heart disease and stroke statistics-2017 update: a report from the American Heart Association *Circulation* **135** e146–603
[3] Masarone D, Kaski J P, Pacileo G, Elliott P M, Bossone E, Day S M and Limongelli G 2018 Epidemiology and clinical aspects of genetic cardiomyopathies *Heart Fail. Clin.* **14** 119–28
[4] Spees J L, Lee R H and Gregory C A 2016 Mechanisms of mesenchymal stem/stromal cell function *Stem Cell Res. Ther.* **7** 125
[5] Liang X, Ding Y, Zhang Y, Tse H-F and Lian Q 2014 Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives *Cell Transplant.* **23** 1045–59
[6] Zhou Y, Yamamoto Y, Xiao Z and Ochiya T 2019 The immunomodulatory functions of mesenchymal stromal/stem cells mediated via paracrine activity *J. Clin. Med. Res.* **8** 1025
[7] Kapur S K and Katz A J 2013 Review of the adipose derived stem cell secretome *Biochimie* **95** 2222–8
[8] Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove C J, Bovenkerk J E, Pell C L, Johnstone B H, Considine R V and March K L 2004 Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells *Circulation* **109** 1292–8
[9] Liguori T T A, Liguori G R, Moreira L F P and Harmsen M C 2018 Fibroblast growth factor-2, but not the adipose tissue-derived stromal cells secretome, inhibits TGF-β1-induced differentiation of human cardiac fibroblasts into myofibroblasts *Sci. Rep.* **8** 16633
[10] Liguori T T A, Liguori G R, Moreira L F P and Harmsen M C 2019 Adipose tissue-derived stromal cells' conditioned medium modulates endothelial-mesenchymal transition induced by IL-1β/TGF-β2 but does not restore endothelial function *Cell Prolif.* **52** e12629
[11] Banovic M, Pusnik-Vrckovnik M, Nakou E and Vardas P 2018 Myocardial regeneration therapy in heart failure: current status and future therapeutic implications in clinical practice *Int. J. Cardiol.* **260** 124–30
[12] Costantino S and Paneni F 2018 Stem cell therapy in heart failure: is the best yet to come? *Int. J. Cardiol.* **260** 135–6
[13] van Dongen J A, Getova V, Brouwer L A, Liguori G R, Sharma P, Stevens H P, van der Lei B and Harmsen M C 2019 Adipose tissue-derived extracellular matrix hydrogels as a release platform for secreted paracrine factors *J. Tissue Eng. Regen. Med.* **13** 973–85

[14] Liguori G R, Liguori T T A, de Moraes S R, Sinkunas V, Terlizzi V, van Dongen J A, Sharma P K, Moreira L F P and Harmsen M C 2020 Molecular and biomechanical clues from cardiac tissue decellularized extracellular matrix drive stromal cell plasticity *Front. Bioeng. Biotechnol.* **8** 520
[15] Sonnenberg S B *et al* 2015 Delivery of an engineered HGF fragment in an extracellular matrix-derived hydrogel prevents negative LV remodeling post-myocardial infarction *Biomaterials* **45** 56–63
[16] Martino M M *et al* 2015 Extracellular matrix and growth factor engineering for controlled angiogenesis in regenerative medicine *Front. Bioeng. Biotechnol.* **3** 45
[17] Garcia J R, Campbell P F, Kumar G, Langberg J J, Cesar L, Wang L, García A J and Levit R D 2017 A minimally invasive, translational method to deliver hydrogels to the heart through the pericardial space *JACC Basic Transl. Sci.* **2** 601–9
[18] de Hilster R H J, Jonker M R, Timens W, Sharma P K, Harmsen M C, Hylkema M N and Burgess J K 2019 Human lung stiffness and viscoelasticity replicated in extracellular matrix hydrogels *Mech. Lung Injury Repair* **54** 586
[19] Tuin A, Kluijtmans S G, Bouwstra J B, Harmsen M C and Van Luyn M J A 2010 Recombinant gelatin microspheres: novel formulations for tissue repair? *Tissue Eng.* A **16** 1811–21
[20] Parvizi M, Bolhuis-Versteeg L A M, Poot A A and Harmsen M C 2016 Efficient generation of smooth muscle cells from adipose-derived stromal cells by 3D mechanical stimulation can substitute the use of growth factors in vascular tissue engineering *Biotechnol. J.* **11** 932–44
[21] Crapo P M, Gilbert T W and Badylak S F 2011 An overview of tissue and whole organ decellularization processes *Biomaterials* **32** 3233–43
[22] Liu D, Zhang X-L, Yan C-H, Li Y, Tian X-X, Zhu N, Rong J-J, Peng C-F and Han Y-L 2015 MicroRNA-495 regulates the proliferation and apoptosis of human umbilical vein endothelial cells by targeting chemokine CCL2 *Thromb. Res.* **135** 146–54
[23] Feng B, Chen S, Gordon A D and Chakrabarti S 2017 miR-146a mediates inflammatory changes and fibrosis in the heart in diabetes *J. Mol. Cell. Cardiol.* **105** 70–6
[24] Ding Y-F, Peng Y-R, Shen H, Shu L and Wei Y-J 2016 Gualou Xiebai decoction inhibits cardiac dysfunction and inflammation in cardiac fibrosis rats *BMC Complement. Altern. Med.* **16** 49
[25] Wang X-T, Gong Y, Zhou B, Yang J-J, Cheng Y, Zhao J-G and Qi M-Y 2018 Ursolic acid ameliorates oxidative stress, inflammation and fibrosis in diabetic cardiomyopathy rats *Biomed. Pharmacother.* **97** 1461–7
[26] Fang L, Ellims A H, Beale A L, Taylor A J, Murphy A and Dart A M 2017 Systemic inflammation is associated with myocardial fibrosis, diastolic dysfunction, and cardiac hypertrophy in patients with hypertrophic cardiomyopathy *Am. J. Transl. Res.* **9** 5063–73
[27] Chao J *et al* 2016 Role of MCPIP1 in the endothelial-mesenchymal transition induced by silica *Cell. Physiol. Biochem.* **40** 309–25
[28] Kim J, Kim J, Lee S H, Kepreotis S V, Yoo J, Chun J-S, Hajjar R J, Jeong D and Park W J 2016 Cytokine-like 1 regulates cardiac fibrosis via modulation of TGF-β signaling *PloS One* **11** e0166480
[29] Soldano S, Paolino S, Pizzorni C, Trombetta A C, Montagna P, Brizzolara R, Corallo C, Giordano N, Sulli A and Cutolo M 2017 Dual endothelin receptor antagonists contrast the effects induced by endothelin-1 on cultured human microvascular endothelial cells *Clin. Exp. Rheumatol.* **35** 484–93
[30] Cai L, Dewi R E, Goldstone A B, Cohen J E, Steele A N, Woo Y J and Heilshorn S C 2016 Regulating stem cell secretome using injectable hydrogels with *in situ* network formation *Adv. Healthc. Mater.* **5** 2758–64
[31] Robert A W, Azevedo Gomes F, Rode M P, Marques da Silva M, Veleirinho M B D R, Maraschin M, Hayashi L, Wosgrau Calloni G and Stimamiglio M A 2019 The skin regeneration potential of a pro-angiogenic secretome from human skin-derived multipotent stromal cells *J. Tissue Eng.* **10** 2041731419833391
[32] García-Olivas R, Hoebeke J, Castel S, Reina M, Fager G, Lustig F and Vilaró S 2003 Differential binding of platelet-derived growth factor isoforms to glycosaminoglycans *Histochem. Cell Biol.* **120** 371–82
[33] Thompson S, Martínez-Burgo B, Sepuru K M, Rajarathnam K, Kirby J A, Sheerin N S and Ali S 2017 Regulation of chemokine function: the roles of GAG-binding and post-translational nitration *Int. J. Mol. Sci.* **18** 1692