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**Label-free relative quantification of secreted proteins as a non-invasive method for the quality control of chondrogenesis in bioengineered substitutes for cartilage repair**

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## **ABSTRACT:**

Cartilage tissue engineering is making progress but the competing available strategies still leave room for improvement and consensual overviews regarding the best combinations of scaffolds and cell sources are limited by the capacity to compare them directly. In addition, because most strategies involve autologous cell transfer, once these will be optimized, the resulting implants will require individual quality control prior to grafting in order to emphasize patient-to-patient differential responsiveness to engineering processes. Here, the limits and advantages of analyzing, by label-free liquid-chromatography-coupled MALDI mass spectrometry (LC-MALDI), the secreted proteome released into culture medium by engineered cartilage tissues, were investigated and compared to more classically used methods for biomaterial characterization. This method did not require sacrificing the biomaterials and robustly evidenced their chondrogenic statuses. In more details, the method highlighted differences between batches prepared from distinct donors. It was adapted to distinct scaffolds and allowed comparing the influence of individual engineering steps, such as growth factor combinations and oxygen tension. Finally, it evidenced subtle changes between replicate substitutes within a series, thereby distinguishing least and most accomplished ones. We conclude that relative quantification of secreted proteins through label-free LC-MALDI will be useful not only to orientate engineering methodologies but also to ultimately provide non-invasive quality control of engineered tissue substitutes for the repair of cartilage and possibly other connective tissues.

## **1. Introduction**

Due to its strictly avascular nature and low propensity to cellular regeneration, joint cartilage is prone to functional degeneration upon long term mechanical challenge or acute trauma. Tissue engineering aims to replace damaged tissues with synthetic tissue constructs grown *in vitro* to mimic the composition and properties of tissues in a controlled laboratory environment. In particular, cartilage constructs designed for surgical repair of focal lesions are considered as promising perspectives. A variety of combinations of synthetic scaffolds and/or cell sources (Huang et al. 2016), together with cultivation methods and additives to induce proper implant maturation have been explored over the last decade, without reaching a consensus regarding best adapted methods. One reason why, is that comparing all proposed strategies in a single experiment requires expertise in all of them, which is difficult to achieve.

Once a strategy is mature, it would be preferable to benefit from preimplantation quality control of the constructs for optimal results. Various techniques, including histology, immunohistochemistry, quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) allow characterizing gene expression and gross extracellular matrix content of tissue-engineered substitutes. However, these techniques require sacrificing the sample and therefore, do not allow characterizing the exact substitute that is considered for implantation. This may be a strong limitation in terms of pre-surgical quality control if, despite efforts in minimizing them, significant differences remain between replicate implants. In addition, these methods focus on very specific and therefore limited aspects of the chondrogenic phenotype and a more subtle definition of the chondrogenic state of the implants is needed. Finally, most of these methods require several days, at best, for technical processing and data interpretation.

The secretome consists in the population of proteins produced by cells and released into the culture medium. It has been used to characterize the chondrogenic differentiation of mesenchymal stem cells (MSCs) using mass spectrometry combined to metabolic labelling (Rocha et al. 2014; Rocha et al. 2012). However, metabolic labelling can difficultly be used in routine experiments or be considered non-invasive. Here, the pertinence of using label-free secretome characterization based on Liquid Chromatography-coupled Matrix-Assisted Laser Desorption and Ionization (LC-MALDI) (Riffault et al. 2015) was investigated in terms of

analytical depth, statistical robustness and coherence with gene expression, histology and immunohistochemistry.

## **2. MATERIALS AND METHODS**

### **2.1 Isolation and expansion of mesenchymal stem cells from human bone marrow**

Bone marrow human mesenchymal stem cells (MSCs) were isolated from patients undergoing total hip arthroplasty. This study was approved by our local Research Institution under the registration number DC-2014-2148. It was conducted in informed patients (written consent, non-opposition declaration) in accordance with the usual ethical regulations in collaboration with our local bone bank (Unité de Thérapie Cellulaire et Tissus, UTCT, CHU Nancy). The average age of donors was 73 years (range 60-80 years). Nucleated cells were counted in the aspirate and plated at a density of  $5 \cdot 10^5$  cells/cm<sup>2</sup> in a T75 culture flask at 37°C in a humidified atmosphere containing 5 % (v/v) carbon dioxide. The medium used from passage 0 to passage 2 was Dulbecco's modified Eagle Medium with low glucose (DMEM-LG, 31885, Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma), 1 ng/ml bFGF (Miltenyi), glutamine and penicillin-streptomycin (Gibco). The medium was changed twice weekly from day 3 and until confluence. Once adherent cells reached about 80 % confluence, MSCs were trypsinized and plated at a density of  $5 \cdot 10^6$  cells/flask. During the last passage before seeding in biomaterials (passage 3), flasks were cultured in differentiation medium composed of DMEM with high glucose, supplemented with 10 % fetal bovine serum, sodium pyruvate (110 µg/ml), bFGF, penicillin-streptomycin, proline (40 µg/ml), L-Ascorbic acid-2-Phosphate (50 µg/ml) and dexamethasone ( $10^{-7}$  M).

### **2.2 General study design**

Cells from 10 patients were used in this study (referred to as batches below). Five batches were used to investigate batch-to-batch reproducibility. Two batches, including one already used in the batch-to-batch reproducibility assay (donor 5), were used to investigate the differential influences of distinct growth factor cocktails. Two independent batches were used to study the influence of the 3D environment and that of hypoxia. Finally, two more were used to investigate intra-series reproducibility. In all experiments, a

minimum of 6 experimental replicates were used, out of which three were used for each analytical method, excepted in the intra-series reproducibility assays, where all 6 replicates were used for secretome analysis, each being analyzed 3 times independently.

### **2.3 Chondrogenic differentiation in collagen sponge**

The biomaterials used in most experiments were type I/III collagen sponges (95% of type I collagen; diameter: 5 mm, thickness: 2 mm) manufactured by Symatèse Biomatériaux (Chaponost). Collagen sponges were inserted into a 96-well plate (one scaffold per well) and seeded by adding  $5 \cdot 10^5$  cells suspended in 50  $\mu$ l DMEM 4.5 g/l glucose containing 10 % FBS and penicillin-streptomycin. The resulting constructs were incubated at 37°C for 4 h to allow cell attachment and were transferred to a 48-well plate containing 1 ml of culture medium already (one construct per well). Sponges were cultured for 28 days in basic medium composed of high-D-glucose (4.5 g/l) DMEM medium supplemented with glutamine, streptomycin and penicillin, proline (40  $\mu$ g/ml, Sigma), L-ascorbic acid-2-phosphate (50  $\mu$ g/ml), dexamethasone ( $10^{-7}$  M), sodium pyruvate (1 mM), ITS+ premix (BD, Insulin-Transferrin-Selenium) supplemented or not with growth factors depending on considered experiments. In most cases, cells were stimulated with TGF $\beta$ 1 at 10 ng/ml. Media were replaced every 2-3 days with 1 ml fresh chondrogenic medium for 26 days. At day 26, samples were rinsed twice in PBS for 5 min, transferred to new culture wells and cultured for 48 h in medium supplemented with albumin-free, Insulin, Transferrin and Selenium supplement (ITS, Sigma) plus other additives depending on experiments (such as growth factors) in the same incubator. This switch of culture medium was verified to have no detrimental effect over cell viability (supplemental data 1).

### **2.4 Secretome analysis**

At day 28, media were collected and frozen at -80°C until analysis. All samples to be compared to each other were analyzed at a same time through label-free relative quantification as described in (Riffault et al. 2015). In each experiment, one reference sample was used for MS/MS measurements of compounds

exhibiting a signal to noise ratio above 20. Then, based on t-test calculations, all compounds found at ratio above 2 compared to the reference sample (in any other sample from the experiment) and not already identified were also processed for MS/MS measurement. Finally, LC-MS/MS runs were merged via WARP-LC software (Bruker) and the whole dataset was used for a single Swissprot database interrogation. Ratios found for all compounds were then exported to the identification list in Proteinscape for peptide and protein ratio calculation as detailed in (Riffault et al. 2015).

## 2.5 Gene expression analysis

At day 28, cells were collected from collagen sponge constructs by collagenase enzymatic digestion for 4 hours and centrifugation. The chondrogenic differentiation of bone marrow MSCs was assessed based on mRNA expression levels of different genes reflecting chondrogenic differentiation of MSCs in cartilage substitutes : *Col2a1* (specific for cartilaginous tissues), *aggrecan* (*Acan*, a large proteoglycan which is a major component of extracellular matrix of cartilaginous tissues), *COMP*, *SOX9* (transcription factor known to play a key role in chondrogenesis), *Colla1* (markers of fibrotic cartilage tissue), *Col10a1* (marker of chondrocyte hypertrophy). In the light of proteomics data, *Matrilin-3* (*MATN3*) was also analyzed *a posteriori*. Total RNA was extracted using the RNeasy mini kit (Qiagen), according to manufacturer's instructions. RNA was quantified spectrophotometrically and reverse transcribed with the iScript cDNA Synthesis Kit (Biorad) and quantitative RT-PCR was performed with a Lightcycler (Roche) using a standard curve ranging from  $10^{-3}$  to  $10^{-6}$   $\mu\text{g/ml}$  for quantification. For each condition, the signal of the *RP29* housekeeping gene was determined once for each cDNA sample, and was used to normalize the expression of others.

## 2.6 Histology

At day 28, collagen sponges were fixed in 4 % paraformaldehyde at pH 7.4 for 24 h, dehydrated and embedded in paraffin wax. 5  $\mu\text{m}$  sections were cut using a sledge microtome and mounted onto glass slides

and stained using Haematoxylin-Erythrosine-Saffron (HES) for morphology and Alcian blue for proteoglycans. Analysis was managed on an optical microscope (DMD108, Leica).

## **2.7 Immunohistochemistry**

Paraffin-embedded tissue sections of 5  $\mu\text{m}$  were deparaffinized and digested with pepsin (0.4 % w/v, Sigma) for 30 min at room temperature prior to immunostaining. Primary antibodies were monoclonal anti-type II collagen (6B3, Labvision) and anti-type I collagen (T59103R, Biodesign). Detection was performed using the LSAB+ kit (HRP, Dako) based on avidin-biotin techniques, following manufacturer's recommendations. Sections were counterstained with hematoxylin (RAL Diagnostics), mounted with Eukitt resin. Pictures were captured using an optical microscope (DMD108, Leica).

## **2.8 Batch-to-batch reproducibility**

MSCs obtained from 5 donors were used in this experiment. Cartilaginous tissue-engineered substitutes were prepared in collagen sponges and randomly assigned to two groups: The one was cultured in basal medium and the other in chondrogenic medium supplemented with TGF $\beta$ 1, the media being replaced every 2-3 days for 28 days. Secretomes and histological/immunohistological analyses were performed from the exact same samples.

## **2.9 Influence of growth factors on chondrogenesis**

MSCs from 2 distinct donors were used for these experiments. To determine the optimal combinations of growth factors, collagen sponge-based substitutes were stimulated during the whole differentiation period with either no additive, TGF $\beta$ 1, TGF $\beta$ 3, BMP2 (Myltenyi, 100 ng/ml), TGF $\beta$ 1 + BMP2 or TGF $\beta$ 3 +BMP2.

## **2.10 Chondrogenic differentiation in alginate beads**

Alginate (Sigma) /hyaluronic acid (HA, Accros) powders were mixed at a weight ratio of 4:1 and autoclaved at 120°C for 20 min for sterilization. In order to create alginate gel cultures, cells were suspended at a



concentration of 3 million cells/ml in 2 % (w/v) viscosity alginate medium prepared in 0.9 % (w/v) NaCl. The cell/alginate preparation was polymerized dropwise into 102 mM CaCl<sub>2</sub> for 10 min. Beads were washed three times with saline and once with basal culture medium. The chondrogenic differentiation medium was the same as for collagen sponges, supplemented with 1 mM CaCl<sub>2</sub>. Secretome analysis was performed strictly as for collagen sponges.

### **2.11 Chondrogenic differentiation in pellet cultures**

Cells were resuspended in the same medium as for collagen sponges at 1 million cells/ml. 1 ml aliquots were dispensed into sterile 15 ml polypropylene tubes and spun for 5 min at 200 g and the resulting pellets were grown within the tubes. Media and secretome analysis were as described for collagen sponges.

### **2.12 Influence of oxygen tension on chondrogenesis**

To determine the effect of hypoxia on the quality of synthesized extracellular matrix, chondrogenic differentiation was performed in the same culture medium but samples were incubated either under normoxia (20 % O<sub>2</sub>) or hypoxia (2 % O<sub>2</sub>).

### **2.13 Intra-series reproducibility**

Two donors were used to study the inter-sample reproducibility between 6 different cartilaginous tissue-engineered substitutes seeded with MSCs from the same donor, prepared as previously described and cultured in chondrogenic medium including TGFβ<sub>1</sub>. For each cartilaginous substitute, three aliquots of conditioned medium were collected and analyzed independently, allowing comparison of the analytical and experimental sources of variability in measurements. Presented data are representative of one experiment only. In the other experiment, due to lower heterogeneity of the data, no significant changes between replicates could be evidenced.

### **2.14 Statistical analysis**

For mass spectrometry, statistics were managed through ProfileAnalysis 2.0 (Bruker), for Principal Component Analysis (PCA) of raw data and group-to-group t-tests calculation for all compounds observed in both groups considered. Relative quantifications per protein were calculated in Proteinscape 2.1 (Bruker) based on the median value of ratios found for all peptides of each given protein. The standard deviation indicated for each protein level (SD) was that observed between all corresponding peptides. Therefore, the SD is indicative of the precision in ratio measurements, but not of the variations observed between replicates. The significance of protein changes was estimated based on the protein p-value, obtained by averaging the p-values found for all peptides used for its quantification (Riffault et al. 2015).

In gene expression studies, values were expressed as average of replicates, + SD. P-values were those of the unilateral Student t-test considering equal variances.

### **3. RESULTS**

#### **3.1 The need for a quality control in biomaterials development: batch-to-batch variations in the quality of implants**

Ideally, an optimized tissue-engineering process should provide implants with optimal and therefore indistinguishable properties. However, when using autologous cell transplantation, intrinsic differences between patients influence the characteristics of biomaterials despite the use of strictly similar engineering protocols. Here, we compared tissue-engineered cartilage implants built from MSCs obtained from 5 distinct donors through a same protocol and characterized the resulting implants at a histological level (figure 1) and at a molecular level (supplemental data 2). Although strictly identical engineering processes were used for all 5 donors, histological and immunohistological analysis demonstrated dramatic distinctions between end-point biomaterials: For 3 donors out of 5 (donors 1, 2, and 3), cartilaginous tissue engineered substitutes exhibited a homogeneous extracellular matrix rich in proteoglycans (PGs) and type II collagen (COL2A1) within collagen sponges stimulated with TGF $\beta$ 1 (figure 1). One of the 3 appeared to contain a higher level of PGs and type II collagen (COL2A1), based on staining intensities (donor 1, figure 1). For the two remaining donors (donors 4 and 5), the sponge content in PGs and COL2A1 was much more heterogeneous, localizing mainly to the most outer part of sponges, and overall, much weaker than for the first three (figure

1). At the gene expression level (supplemental data 2), overall, the addition of TGF $\beta$ 1 led to a dramatic increase of chondrogenesis markers. Expression of cartilage specific genes, such as *Col2a1*, *SOX9*, *ACAN*, *COMP*, *Col10a1* were highly increased with TGF $\beta$ 1, in agreement with its chondro-inductive properties towards MSCs. In the case of *Col2a1*, expression levels appeared to be correlated to immunohistological results in the sense that donors 4 and 5 exhibited significantly lower levels than others, yet with disappointing statistics. However, other investigated genes did not allow distinguishing best from poorest responders at all. Altogether, mRNA data were efficient at evidencing the influence of TGF over chondrogenesis, but did not demonstrate dramatic distinctions between donors, in disagreement with histological and immunochemical data.

### **3.2 Secretome analysis provides an overview of the chondrogenic status that is coherent with classical analytical methods**

In order to emphasize the pertinence of secretome analysis as readout of the quality of implants, secretomes of triplicate sponges obtained from all 5 donors were analyzed and compared to each other (figure 2). As expected, all protein levels found measurable correlated well to mRNA data when available, in the sense that the stimulation by TGF $\beta$ 1 led to dramatic increases of all major chondrogenic markers (not shown here but evidenced farther down). When comparing secretomes between distinct donors, most proteins did not exhibit significant, recurrent differences. For instance, Aggrecan (PGCA) and COMP were not particularly informative, while they are recurrently used as chondrogenesis markers at the mRNA level. Also, type I collagen (COL1A1 and COL1A2), which is considered as marker of a poor chondrogenesis, did not exhibit significant differences. On the contrary, COL2A1, Fibromodulin (FMOD), the  $\alpha$ 2 chain of type XI collagen (COL11A2), proenkephalin (PENK) and Matrilin-3 (MATN3), which were all reported as mid-late to late chondrogenic differentiation markers, respectively (Xu et al. 2008), were all found significantly decreased in donors 4 and 5 compared to others. *Matn3* was verified *a posteriori* to distinguish both groups of donors also at the gene expression level (supplemental data 2), strengthening the findings. Finally, Biglycan (PGS1, (Barry et al. 2001)), which was reported as an early differentiation marker, was significantly increased in donors 4 and 5 compared to others. Therefore, most proteins measured in secretome studies were

representative of the differentiation state of biomaterials and several of them allowed distinguishing batches of bioengineered cartilage tissues depending on their overall quality.

### **3.3 Improving culture methods for optimal chondrogenic differentiation based on secretome characterization**

The above results suggest that secretome analysis allows evidencing the chondrogenic status of biomaterials. However, differences found between donors may reflect the potential of cells obtained from either donor to undergo chondrogenic differentiation, without influence of the detailed protocol used to induce it. Therefore, we investigated the influence of distinct combinations of growth factors, administered during the whole differentiation step, over the content of secretomes (figure 3). Samples were also analyzed at the histology/immunohistochemistry (supplemental data 3) and gene expression (supplemental data 4) levels. Overall, BMP2 by itself was not found to promote chondrogenesis based on secretome analysis, as most relevant proteins remained at levels comparable to the ITS negative control. On the contrary, most of them were largely upregulated by any TGF $\beta$  isoform. Most were found at very comparable levels after stimulation with TGF $\beta$ 1, TGF $\beta$ 3, or either one combined with BMP2. However, a few of the most specific markers of chondrogenesis (COL2A1, COL11A2, COL10A1) were found significantly increased with TGF $\beta$ 3 or combinations of either TGF $\beta$  isoform with BMP2, compared to TGF $\beta$ 1 alone, in agreement with previous reports based on classical analytical procedures (Henrionnet et al. 2010). Therefore, secretome analysis highlighted the differential influences of distinct combinations of growth factors. It is worth mentioning that at the mRNA level (supplemental data 4), differences were significant only for *Col10a1* between TGF $\beta$ 1 and TGF $\beta$ 3. At the immunochemical level (supplemental data 3), an apparent COL2A1 induction with combined growth factors was observed, but difficult to ensure due to the lack of numerical data. A more trustable increase in proteoglycan synthesis observed at the histological level was not evidenced in secretome analysis based on Aggrecan measurements (PGCA). This may suggest that the corresponding samples exhibited increased glycan elongation rather than proteoglycan core protein secretion. Indeed, one remaining limitation of the proposed method for secretome analysis is the inability to investigate glycosylation states.

Overall, however, secretome analysis was more powerful at evidencing the differential influence of subtle growth factor changes than the other methods available.

### **3.4 Secretome analysis allows comparison of distinct 3D-culture methods**

The secretome is considered to reflect the metabolic activity of cells and therefore it should be interpretable independently on the scaffold used for cell seeding. However, distinct culture conditions may interfere distinctively either with secretion itself or with sample preparation and therefore, in practice, secretome analysis may require specific adaptations to each culture condition. Furthermore, the scaffolds influence differentiation itself and this should be revealed through secretome analysis. In order to emphasize both the robustness of the method without adaptations and the influence of culture conditions, secretomes of MSCs undergoing chondrogenic differentiation either in collagen sponges, alginate beads or cell pellets were compared (figure 4). As reflected by the lower number of secreted proteins that could be quantified, the pellet culture system appeared to be less compatible than others with the analytical method. This may be due at least in part to the apparently higher contamination of secretomes by intracellular proteins in this culture system (highlighted in figure 4 by the higher level of actin; ACTB). Still, major proteins relevant of the chondrogenic differentiation remained measurable. Several of these did not exhibit significant differences between culture conditions. However, in several respects, alginate beads appeared more favorable to the differentiation process, as in particular, COL2A1, COL10A1 and COL11A2 were found at significantly higher levels, while the same number of cells was initially seeded in all three culture systems. Of note, in the experiment shown here, alginate beads were also found to secrete more CTGF, a growth factor involved in early stages of chondrogenesis (Song et al. 2007), which had not possibly been identified in the previous experiments, reflecting, again, batch-to-batch differences.

### **3.5 The influence of hypoxia depends on the 3D environment**

One major reason why a consensus regarding the optimal methods to drive the chondrogenic differentiation of MSCs has not been reached is that it is difficult to transpose findings made in a given culture condition to

another. For instance, the influence of hypoxia has not been reported as critical in all culture methods (Bornes et al. 2015). In order to better understand this apparent discrepancy, the influence of hypoxia was investigated in alginate beads, collagen sponges and MSC pellets (figure 5). Indeed, in MSC pellets, hypoxia exhibited little influence over the secretome, while in collagen sponges and even more so alginate beads, it did significantly potentiate secretion of major differentiation markers.

### **3.6 A non-invasive method sufficiently robust to evidence variations between replicate substitutes**

One major advantage of secretome analysis for the characterization of cartilage substitutes is its non-invasive nature, allowing generating a comprehensive overview of the quality of the precise sample that is considered for implantation. This may be very important, notably if replicate substitutes of a given batch do not have exactly the same properties. However, it is not obvious either that the analytical method would allow a sufficiently robust characterization to evidence the differences, if there were any. To reach such an objective, the analytical method in its entirety, that is, including sample preparation and analysis *per se*, should exhibit a sufficiently low intrinsic variability compared to that of the engineering process. In order to better emphasize the potential of secretome analysis in this respect, the variations of the entire process, that is, engineering plus analysis, were compared to those of the analytical method only (figure 6). To do so, the secretomes of 6 collagen sponge substitutes prepared following exactly the same procedure in parallel were split into 3 aliquots each and all aliquots was analyzed separately. In this way, the differences between aliquots of a given substitute were representative of the variations due to the analytical process, while those between aliquots of separate substitutes revealed the summed variations of engineering and analysis. Principal component analysis (PCA) was first used to evidence crude variations within the whole population of variables (the variables being the level at which each molecule was measured by mass spectrometry). The repartition of samples throughout the PCA plot (figure 6. A) suggested that 2 of the 6 experimental replicates should be distinguishable from the rest based on secretome analysis, the one differing from the rest in a direction opposite to the other. Indeed, when comparing levels of individual proteins between experimental replicates, one of these two samples was found to secrete moderately but significantly lower levels of FMOD, COL10A1 and MATN3. Therefore, this sample may be considered as of significantly

suboptimal chondrogenic differentiation. On the contrary, the other distinguishable sample exhibited significantly (but still, moderately) increased COL10A1 and lower COL1A2 and may therefore be considered as of superior differentiation. Altogether, these data indicated that secretome analysis was sufficiently robust to distinguish samples of least and most accomplished chondrogenic differentiation even within a population of experimental replicates. It is worth mentioning, however, that when performed on another batch of substitutes, the method did not allow distinguishing samples from one another, probably because in this experiment, there was a lower intrinsic heterogeneity of samples (data not shown).

#### **4. DISCUSSION**

Tissue engineering for cartilage repair uses a variety of strategies among which the combination of an artificial scaffold with autologous cells driven through functional differentiation is widely studied. However, whenever cell engineering is involved, a method allowing validating the functional state of cell contingents prior to transplantation is required for each independent engineering process. In addition, whenever differential results between engineering replicates are suspected, validation methods need to be non-invasive, allowing the precise analysis of the sample that is to be implanted. Furthermore, for the sake of the patient, in such situations, several replicates should be processed and compared in terms of quality to ensure grafting of the optimally engineered substitute. In the past, we have proposed SHG microscopy as a non-invasive tool to reveal the type II collagen content of biomaterials (Dumas et al. 2010). However, while the proposed method allowed evidencing differences between fully processed biomaterials and negative controls, its statistical interpretation did not allow comparing experimental replicates between them. Here, we investigated the feasibility and pertinence of secretome analysis through label-free relative quantification by LC-MALDI as a quality control method. The principle itself of using the secretome as a quality index of biomaterials may be questioned; many proteins reported here are considered part of the extracellular matrix, such as collagens, COMP, SPRC, proteoglycans, FMOD, MATN3 and some of them turn out to be the most distinctive markers of chondrogenesis in our experiments. Indeed, the deposition of proteins into the extracellular matrix *in vitro* is not an extremely effective process and improving it may be an important aspect of future developments in tissue engineering (Prewitz et al. 2015). Several other proteins reported

here are intrinsically soluble, such as TIMP1, MMP2, IBPs and CTGF. Ideally, one would appreciate measuring levels of additional such soluble factors, notably, Interleukin-6, which is considered as informative of the pro-inflammatory status of the substitute (Morille et al. 2016). It is worth mentioning that this protein and a few others of comparable function have occasionally been observed using the method reported here, but only from samples that did not differentiate optimally (not shown). Indeed, the method used here was specifically focused on chondrocyte-like secretomes and in particular, the details of the liquid chromatography were optimized to use as little machine time as possible without loss of critical information, in an effort to minimize analytical costs and the waiting period until end results. At present, the method can provide definitive quality control results within less than 72 hours on a routine basis when comparing no more than 32 samples.

The method was found efficient at discriminating batches of biomaterials that exhibited limited quality compared to others (figure 2). Secretome analysis revealed or confirmed both positive and negative markers of differentiation. It may be tempting now to use these markers in ELISA-style assays rather than performing mass spectrometry to investigate the secretomes of biomaterials. However, there remains much room for the improvement of cartilage substitutes and, in the course of upcoming evolutions, additional, possibly more pertinent markers may be revealed. In this context, one major advantage of secretome analysis through mass spectrometry in the follow up of engineering processes is that the list of markers evolves incrementally, because the analytical process does not target particular proteins, but rather, measures all that there is to be measured, the only limit being sensitivity.

A good example of possibly useful and yet difficultly anticipated marker, found here to discriminate substitute batches (figure 2) was proenkephalin (PENK). Although already reported as a late marker of chondrogenesis (Xu et al. 2008), PENK has no known function in cartilage biology, but rather in nociception (Konig et al. 1996). However, its levels reached the sensitivity threshold only in particular sets of experiments. All other markers found here were directly or indirectly reported before. For instance, although type XI collagen is considered a late marker of chondrogenesis (Xu et al. 2008), it is rarely used in practice as a marker in tissue engineering. Type XI collagen is a heterotrimer composed of COL11A1, COL11A2 and COL2A1 chains. Intuitively, when willing to follow type XI collagen synthesis, one may



refer to levels of COL11A1. However, in our whole dataset, COL11A1 was never found as a discriminating marker (see figures 2-5). On the contrary, COL11A2 varied significantly in all experiments. Furthermore, when comparing the effects of various growth factor cocktails (figure 3), COL11A2 was the only variable that significantly distinguished the effect of TGF $\beta$ 3 from that of TGF $\beta$ 1. In many cases, COL11A2 secretion appeared to be correlated to that of COL2A1 (figure 2-5). This is in agreement with their reported partial co-regulation at the gene expression level (Bridgewater et al. 1998). Overall, COL11A2 is revealed here as an informative marker, which could most likely not have been highlighted without a *de novo* marker search.

Other known markers of chondrogenesis were found significantly modulated here, such as FMOD and MATN3 (Xu et al. 2008). However, MATN3 was often close to the limit of measurability and therefore, could not be quantified in the experiment comparing secretomes depending on added growth factors (figure 3). In the experiment that compared culture systems, neither FMOD nor MATN3 could be measured in secretomes obtained from pellet cultures (figure 4). Indeed, in this culture system, fewer proteins could be quantified (figure 4) and this may be considered as a limit of secretome analysis. However, it is remarkable that the only proteins found at higher levels in pellet culture secretomes than in others were not related to the chondrogenic phenotype and occasionally, not even supposed to be secreted (in particular, Actin-B, ACTB), reflecting either the low extracellular matrix synthesis by cells grown in pellets, or a relatively high cell death. Therefore, the low number of proteins found quantifiable with this model may reflect better a limitation of the culture system than one of the analytical method. Indeed, in this model, very few chondrogenesis markers were found increased by hypoxia (figure 5), which is now a well-accepted pro-chondrogenic factor. On the contrary, in collagen sponges and even more so in alginate beads, hypoxia did induce several chondrogenesis-specific markers very significantly.

Our study demonstrates that secretome analysis is useful to monitor the chondrogenic status in a variety of tissue engineering contexts: It allows revealing differences between batches (figure 2), the influence of growth factors and combinations thereof (figure 3), that of culture systems (figure 4) and that of oxygen tension (figure 5). Therefore it is an interesting alternative analytical method to determine optimal cartilage tissue engineering processes. We used only bone marrow MSCs in this study. However, it is difficult to conceive why the method would be less adapted to other cell sources. Although we did not transpose the

method to other tissues, one would speculate that it would also be valuable for the engineering of other artificial connective tissues.

Finally, the method can be used not only to optimize engineering protocols, but also to reveal specificities of individual substitutes within a population of replicates (figure 6), without destroying the considered samples. There again, the proteins found discriminating were mostly the same ones as in our previous experiments (figure 2-5). The presented method offers the possibility to provide a quality control of the very specific samples that are to be grafted and to select the best available substitutes within a series, based on the measurement of multiple parameters, re-enforcing statistical robustness of the quality control. Its principles may in this respect turn out very useful in the upcoming developments of bioengineering.

**Conflicts of interest:** none.

## **5. ACKNOWLEDGEMENTS**

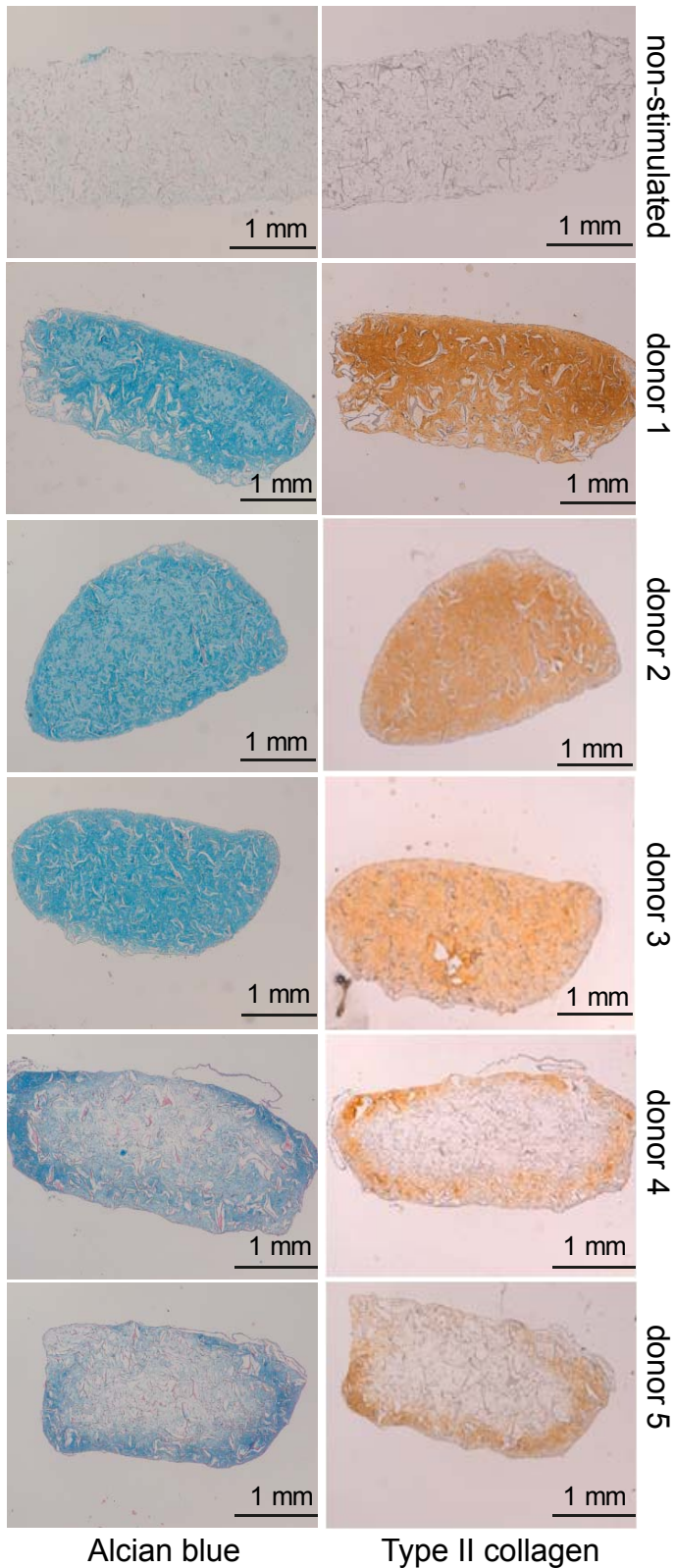
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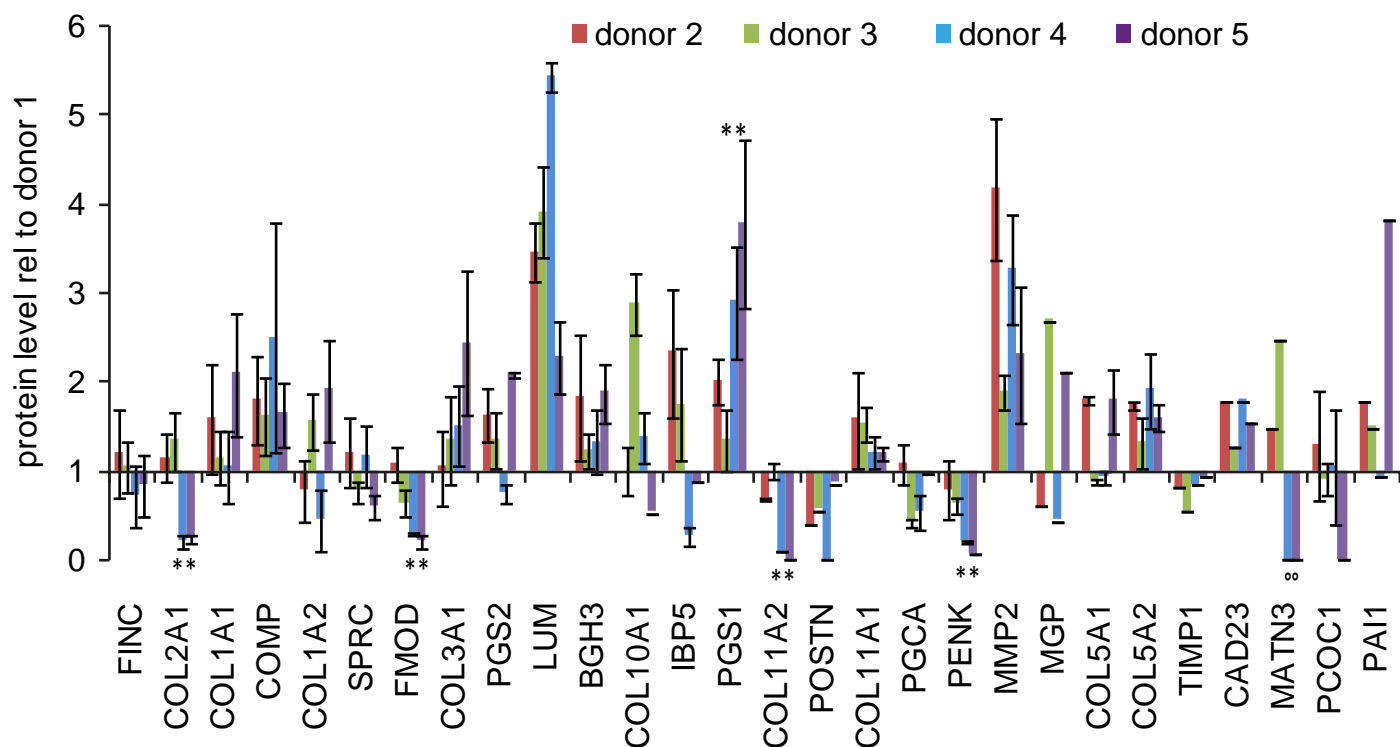
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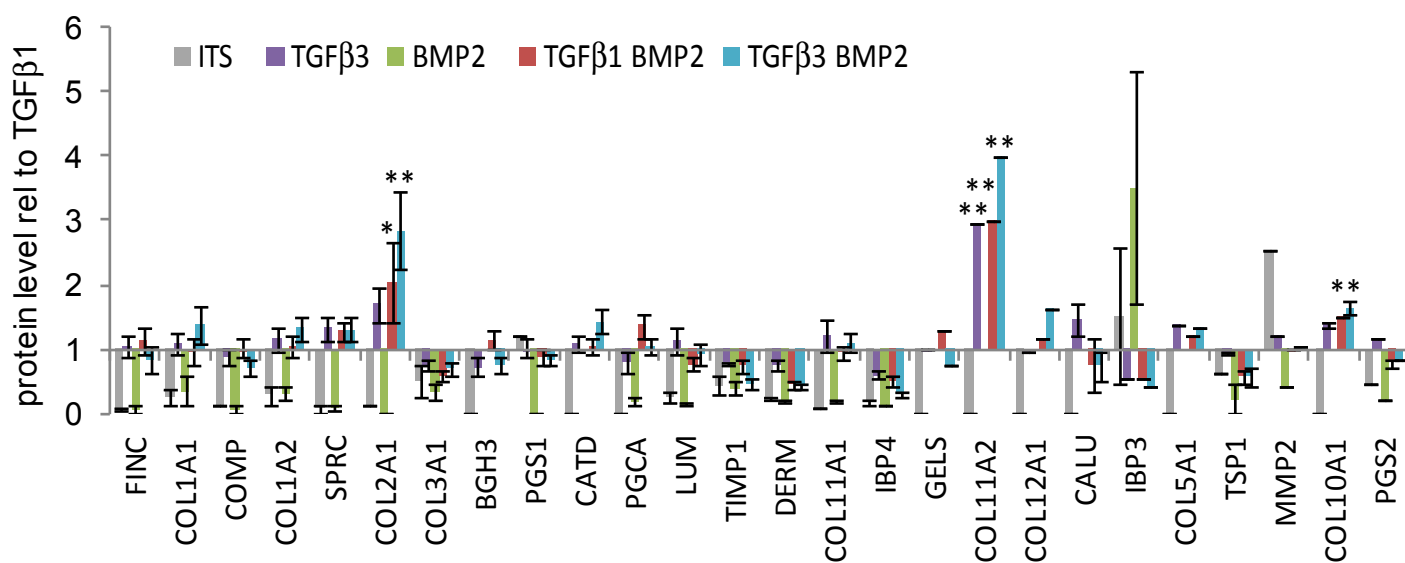


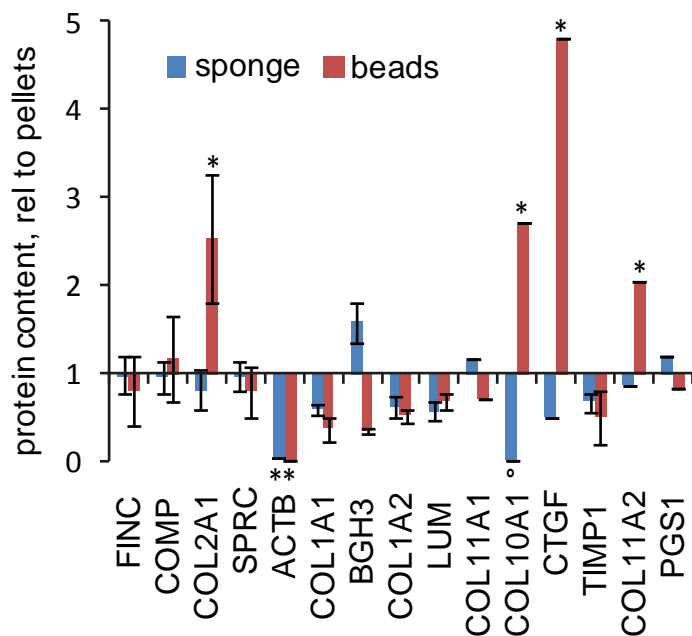
**Figure 1. Histological comparison of sponges obtained from the 5 donors.** Typical results obtained at day 28 without or with TGF $\beta$ 1 stimulation. Sections were taken at D28 from halfway through the depth of the construct were stained for Alcian Blue (stains GAG) and type II collagen by immuno-histochemistry. The non-stimulated sample shown here was from donor 2 but was representative of other donors as well. The numbering of donors does not correspond to their chronological order of processing. Original magnification x4.

**Figure 2. Secretome comparison of sponges obtained from the 5 donors.** Bone marrow mesenchymal stems cells were obtained from 5 distinct donors and then processed through strictly similar maturation processes in collagen sponges. Proteins are listed per decreasing order of Mascot identification score. Values for each protein (Swissprot abbreviated names) are expressed as median value, relative to donor 1, considered here as reference. Bars, SD; \*\*, indicates average protein p-value for donors 4 and 5 < 0.05 versus donor 1; ° indicates that the protein was not detectable for donors 4 and 5.



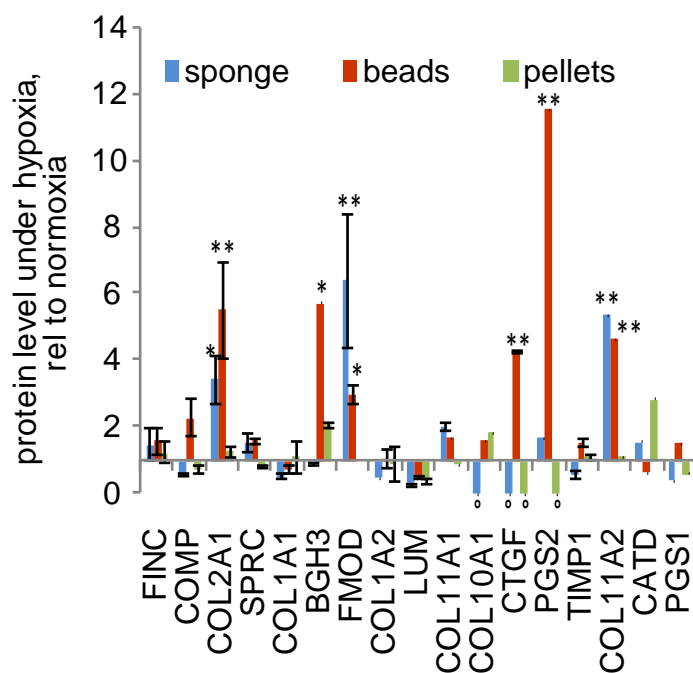
**Figure 3. Secretome comparison of sponges from a same donor but induced to undergo chondrogenesis with distinct growth factor cocktails.** Proteins are listed per decreasing order of Mascot identification score. Values for each protein (Swissprot abbreviated names) are median ratios, relative to those found with TGF $\beta$ 1 stimulation, considered here as reference. Bars, SD; \*, indicates average protein p-value versus TGF $\beta$ 1 < 0.1; \*\* indicates p-value < 0.05. Note: significance of changes for negative controls (ITS or BMP2 alone) is not highlighted for the sake of clarity.



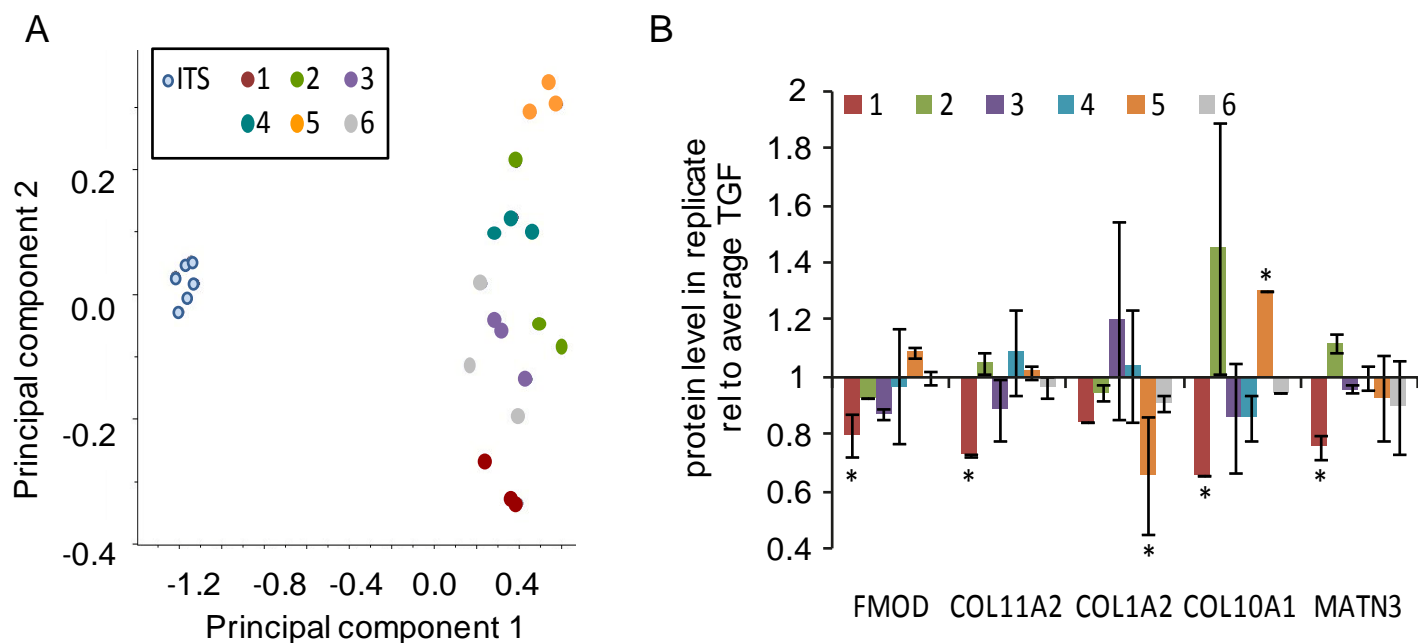


**Figure 4. Secretome comparison depending on culture methods.** Cells from a same donor were processed for chondrogenic differentiation in either cell pellets, collagen sponges, or alginate beads, in the same culture medium. A same number of cells was used for each sample. Proteins are listed per decreasing order of Mascot identification score. Values for each protein (Swissprot abbreviated names) are expressed as median value, relative to the level of pellets, considered here as reference. Bars, SD; \*, indicates average protein p-value < 0.05 versus pellets; °, indicates protein undetectable. Note: the number of proteins reported here is lower because the secretome of pellets, used as reference, is poorer than those of collagen sponges and alginate beads.





**Figure 5. The influence of hypoxia depending on the culture methods.** Cells from a same donor were processed for maturation in either culture system and either under normoxia or hypoxia, in the same culture medium. Proteins are listed per decreasing order of Mascot identification score. Values for each protein (Swissprot abbreviated names) are expressed as median value of levels found under hypoxia, relative to the level found under normoxia. Bars, SD; \*, indicates average protein p-value < 0.1 versus normoxia; \*\*, indicates p < 0.05; °, indicates protein undetectable.



**Figure 6. Statistical analysis of the variations found in secretomes from replicate substitutes.**

This experiment was performed from 6 replicate substitutes made from collagen sponges with TGF $\beta$ 1 stimulation and 2 unstimulated controls. For each sample (indicated by distinct colours), the secretome was split into 3 aliquots and each aliquot was processed for analysis. (A) Principal component analysis of the whole experiment, demonstrating the capacity of the analytical method to discriminate at least some of the replicates from the rest of the population. (B) Values of each protein are expressed as median value, relative to the average level found in all 6 stimulated samples. Bars, SD; \*, indicates average protein p-value < 0.1 versus average. Only proteins for which at least one replicate demonstrated a significant variation compared to the mean are shown.