

Article

Dose-Response Tendon-Specific Markers Induction by Growth Differentiation Factor-5 in human bone marrow and umbilical cord Mesenchymal Stem Cells

Maria C. Ciardulli^{1#}, Luigi Marino^{1#}, Erwin P. Lamparelli¹, Maurizio Guida², Nicholas R. Forsyth³, Carmine Selleri¹, Giovanna Della Porta^{1#§}, Nicola Maffulli^{1,4§}

¹ Department of Medicine, Surgery and Dentistry “Scuola Medica Salernitana”, University of Salerno, Via S. Allende, 1, 84084 Baronissi (SA), Italy; gdellaporta@unisa.it

² Department of Neuroscience and Reproductive Science and Dentistry, University of Naples “Federico II”, Via Pansini, 5, 80131 Naples, Italy; maurizio.guida@unina.it

³ Guy Hilton Research Centre, School of Pharmacy and Bioengineering, Keele University, Stoke-on-Trent, Staffordshire, ST4 7QB, United Kingdom; n.r.forsyth@keele.ac.uk

⁴ Queen Mary University of London, Barts and the London School of Medicine and Dentistry, Centre for Sports and Exercise Medicine, Mile End Hospital, 275 Bancroft Road, London E1 4DG, England, UK; n.maffulli@qmul.ac.uk

* Correspondence: gdellaporta@unisa.it; Tel-fax: +39089965234

The authors equally contributed to the work.

§ The authors shared the co-last authorship.

Abstract: Mesenchymal Stem Cells derived from bone marrow (hBM-MSCs) are utilized in tendon tissue-engineering protocols while extra-embryonic cord-derived, including from Wharton’s Jelly (hWJ-MSC), are emerging as useful alternatives. To explore the tenogenic responsiveness of hBM-MSCs and hWJ-MSCs to hGDF-5 we supplemented each at doses of 1, 10, and 100 ng/mL and determined proliferation, morphology and time-dependent expression of tenogenic markers. We evaluated expression of Collagen types 1 (COL1A1) and 3 (COL3A1), Decorin (DCN), Scleraxis A (SCX-A), Tenascin-C (TNC) and Tenomodulin (TNMD) noting the earliest and largest increase with 100 ng/mL. With 100 ng/mL, hBM-MSCs showed upregulation of SCX-A (1.7-fold) at day 1, TNC (1.3-fold) and TNMD (12-fold) at Day 8. hWJ-MSCs, at the same dose, showed up-regulation of COL1A1 (3-fold), DCN (2.7-fold), SCX (3.8-fold) and TNC (2.3-fold) after 3 days of culture. hWJ-MSCs also showed larger proliferation rate and marked aggregation into a tubular shaped system at Day 7 (with 100 ng/mL of hGDF-5). Simultaneous to this we explored expression of pro-inflammatory (IL-6, TNF, IL-12A, IL-1 β) and anti-inflammatory (IL-10, TGF- β 1) cytokines across for both cell types. hBM-MSCs exhibited a better balance of pro-inflammatory and anti-inflammatory cytokines upregulating IL-1 β (11-fold) and IL-10 (10-fold) at Day 8; hWJ-MSCs, had a slight expression of IL-12A (1.5-fold) but a greater up-regulation of IL-10 (2.5-fold). Collagen type I and tenomodulin proteins, detected by immunofluorescence, confirming the greater protein expression when 100 ng/mL were supplemented. In the same conditions, both cell types showed specific alignment and shape modification (fibroblast-like) with a Length/Width ratio increase at value higher than 1, suggesting their response in activating tenogenic commitment events, and they both potential use in 3D *in vitro* tissue engineering protocols.

Keywords: Wharton’s Jelly human umbilical cord mesenchymal stem cells (hWJ-MSCs); Growth Differentiation Factor-5, human bone marrow Mesenchymal Stem Cells (hBM-MSCs), tenogenic commitment, gene expression, immunofluorescence assay.

1. Introduction

Tendon injuries affect the elderly, active workers, and athletes resulting in marked economic healthcare and societal burden [1][2]. Clinical manifestation itself being the result of a long-term interplay of inflammatory and failed healing response changes [3]. The management of tendon injuries is challenging, given their limited healing capability, and propensity for a failed healing response [4][5]. Anti-inflammatory drugs are frequently used, but they may actually hinder recovery [6], while in clinical practice, tissue grafts or prostheses are used for severe injuries [7][8] and cell therapies proposed for future application [9][10][11].

Multipotent Mesenchymal Stem Cells (MSCs) are described as a promising therapeutic tool to manage tendon conditions in clinical applications [12]. MSCs-based cell therapy exerts beneficial effects in several animal models, mostly mediated by immunomodulatory and trophic mechanisms [13][14][15][16][17][18] where proof of MSCs engraftment is often lacking. MSCs reside in virtually all tissues, in close proximity to the vasculature, [19][20] however, the differentiation capabilities of MSCs can be context-sensitive and their role in tendon remains poorly understood [3]. The use of MSCs in combination with specific growth factors (GFs) is proposed as an innovative treatment for tendon healing and regeneration [21][22]. Human bone marrow MSCs (hBM-MSCs) have been extensively characterized [23]. hBM-MSCs are multipotent able to differentiate into various types of mesenchymal cell phenotypes, including osteoblasts, chondroblasts, myoblasts and tenocytes under specific conditions *in vitro* [24][25][26]. Their application in ligament and tendon reconstruction strategies is promising. They are the most frequently used stem cell type to be explored in tendon repair and can be transplanted to various tissue injury sites, with enhanced tissue repair being achieved [27].

A number of different GFs are involved in tendon repair and in tendon tissue engineering developments [28][29][30]. Growth Differentiation Factor 5 (GDF-5) induces the expression of genes linked to the neo-tendon phenotype [31][32][33] and its deficiency delays Achilles tendon healing in mice [34][35]. However, little is known about the effect of GDF-5 on BM-MSCs transcriptional regulation and differentiation [26]. Park et al., reported that GDF-5 supplemented (100ng/mL) culture media enhanced extracellular matrix (ECM) and tenogenic marker gene expression in adipose-derived mesenchymal stem cells (ADMSCs) over 12 days [36]. GDF-5 also appeared to induce tenogenic differentiation of human BM-MSCs when used at concentration of 100 ng/ml as reflected by significant increases in total collagen expression and tenogenic marker gene expression (Scleraxis, Tenascin-C and type 1 Collagen at day 7) [33]. Further, Bone Morphogenic Protein-14 (BMP-14, also known as GDF-5) (50-100 ng/mL) significantly increased tendon marker expression (Scleraxis and Tenomodulin) at mRNA and protein level potentially via the Sirt1-JNK/Smad1-PPAR γ signaling pathway and enhanced by TGF- β 3 and VEGF [26][37]. Pathway analyses on hBM-MSCs treated with GDF-5 highlighted that the potential molecular pathways involved in GDF-5 induced tenogenic differentiation included cytoskeleton reorganization, cell adhesion, and extracellular matrix signaling [33]. Further investigation demonstrated apparent cytoskeleton reorganization, suggesting an important event in tenogenic differentiation [38]. The association of BMP-14 with enhanced the tenogenic differentiation of BM-MSCs.

hBM-MSCs have been applied within 3D *in vitro* models of tendon healing and regeneration [39][40][41]. However, given the limited number of hBM-MSCs available for autologous use, donor site morbidity, and their limited proliferative capacity, it is important to identify alternative MSCs sources for clinical use and application in tissue engineered protocols of tendon regeneration.

An alternative tissue source of MSCs is the connective tissue (Wharton's Jelly) of the human umbilical cord (hWJ-MSCs) [42][43]. MSCs derived from extra-embryonic tissue, including hWJ-MSCs, share several characteristics with adult MSCs but also retain some features of developmentally immature stem cell populations i.e. broad germ layer-spanning differentiation potential, but do not induce teratomas formation and have a potential application in regenerative medicine that is not impeded by ethical or legal issues [44]. Moreover, hWJ-MSCs display high proliferation rates, wide multipotency, hypoinmunogenicity and have unique anticancer properties, all important features in cell based therapies [45][44]. hWJ-MSCs have been studied for application in neurological disorders [46], kidney injury [47], lung injury [48], orthopaedic injury [49], liver injury [50], and cancer therapy [51] this far. Mesenchymal stromal cells isolated from Wharton's Jelly

have been induced to form neurons, myocytes, bone, cartilage and adipose cells [52][53][54][43][28], but very little is known about their tenogenic commitment.

The repair process in injured tendons consists of three overlapping phases reported as the inflammatory, proliferative and remodeling stages [55]. Several cell types are involved in tendon healing. Pro-inflammatory (M1) and anti-inflammatory (M2) macrophages directly orchestrate tendon remodeling and by secreting cytokines and growth factors, activate the epithelial-to-mesenchymal transition (EMT) signaling cascades in the epithelial cells that surround tendon tissue, providing a source of mesenchymal cells able to repair the injured tissue [55]. Indeed, preclinical and clinical studies have demonstrated the anti-inflammatory and immunomodulatory potential of MSCs, even though the mechanisms behind the MSC-based immunomodulation remain a challenge. For example, MSCs can have different immunomodulatory effects, even on the same types of immune cells, depending on the disease status. However, both hBM-MSCs and hWJ-MSCs display immunomodulatory properties and produce large amounts of cytokines and growth factors, connected to the differentiation processes [56][57][44]. A correlation between GDF-5 and cytokine expression has been noted in human annulus cells in *in vitro* disc degeneration models where high levels of two pro-inflammatory cytokines (IL-1 β and TNF- α) leads to a significant down-regulation of GDF-5 [58].

Determining stem cell responses to specific dosages of GDF-5 treatment is a fundamental step in addressing tendon regeneration strategies, tissue engineering models, and protocols. Here, we report the effect of a range of human GDF-5 (hGDF5) concentrations on tendon and cytokine gene expression on hWJ-MSCs. hBM-MSCs are included to enable direct comparison with the research standard. Markers such as type 1 collagen (COL1A1), Decorin (DCN), Scleraxis A (SCX-A), Tenascin-C (TNC) and Tenomodulin (TNMD), and both pro-inflammatory (IL-6, TNF, IL-12A, IL-1 β) and anti-inflammatory (IL-10, TGF- β 1) cytokines were monitored by gene expression by Real Time Polymerase Chain Reaction (RT-PCR). Type 3 collagen (COL3A1) was used as a negative marker, because it is basal expressed by stem cells. Morphometric analysis with cells alignment, shape and length/width ratio coupled with COL1A1 and TNMD proteins detection by quantitative immunofluorescence completed the proposed study. Key finding from the proposed data was the possible use of hWJ-MSCs, instead of the gold standard hBM-MSCs, in tissue engineering protocols for tendon healing and repair.

2. Results

Detailed flow cytometry characterization of both hBM-MSC and hWJ-MSC is reported in **Table 1** and data acquisition profiles illustrated in **Figure 1**. All samples were positive for CD90, CD105, CD73 and negative for CD14, CD34, CD45, HLA-DR in accordance with previously published data [59]. Proliferation of both cell types was determined during the experimental time course both with and without hGDF-5 supplementation (**Figure 2**). No important statistical significant differences in proliferative potential were noted for hBM-MSCs with hGDF5 supplementation (at Day 16 NT it looks like cells number decreased though) while hWJ-MSCs displayed significant increases in proliferation with both 10 and 100 ng/mL of hGDF-5.

Compared to undifferentiated mesenchymal stem cells, whose nucleus is roundish, the overall tenocytes shape and their nucleus appears elongated with a nuclei length/width ratio larger than 1 [60]. Therefore, hBM-MSCs phenotype commitment was evaluated by nuclear aspect ratio evolution (length/width) determined at day 1, 8 and 16 (**Figure 3a-c**). hBM-MSCs showed specific alignment and shape modification (fibroblast-like) with an L/W ratio increase at value higher than 1 at Day 16, but larger values statistical significant were observed only when 100 ng/mL of hGDF-5 were supplemented. Indeed, in this condition, the average nuclei aspect ratio (length versus width of the nucleus) at days 8 and 16 showed statistically significant differences and a mean increment of 1.4-fold.

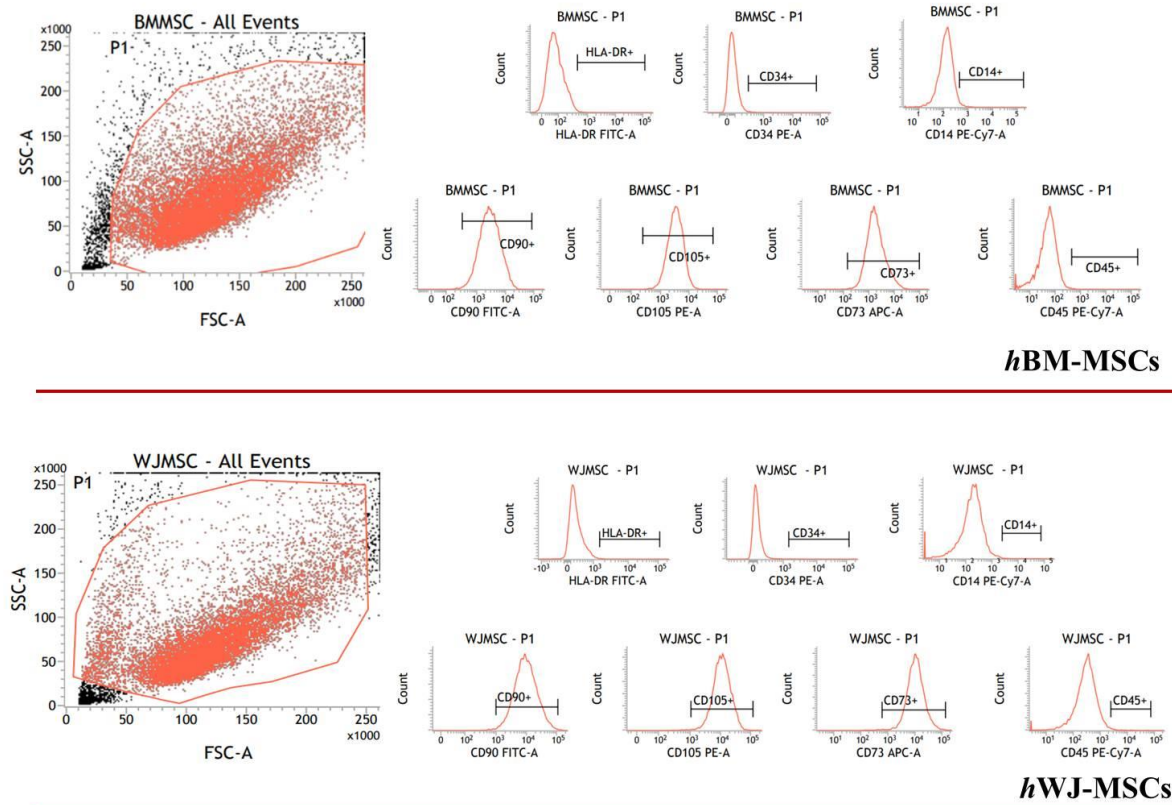


Figure 1. Flow cytometry events illustrating hBM-MSCs and hWJ-MSCs characterization. The panel shows the representative flow cytometry event of FSC vs SSC. The cells were positive for CD90, CD105, CD73 and negative for CD14, CD34, CD45, HLA-DR before use.

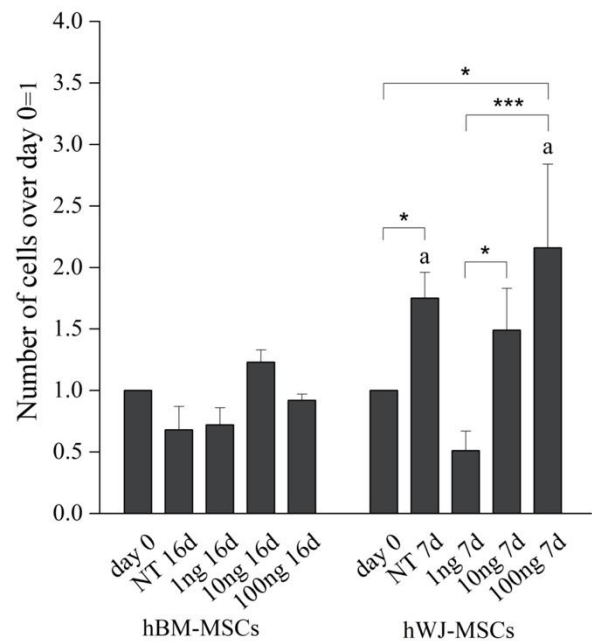


Figure 2. Proliferation rate evaluated on hBM-MSCs and hWJ-MSCs over culture time points. hBM-MSCs not proliferate during the treatment, whereas, hWJ-MSCs showed a statistically significant proliferation with both 10 and 100 ng/mL of hGDF-5 treatment. Statistically significant differences are shown as * = $p < 0.05$; ** = $p < 0.01$; *** = $p \leq 0.005$; a = $p < 0.05$ compared to hBM-MSCs (NT 7d vs NT 16d; 100ng 7d vs 100ng 16d); $n=3$.

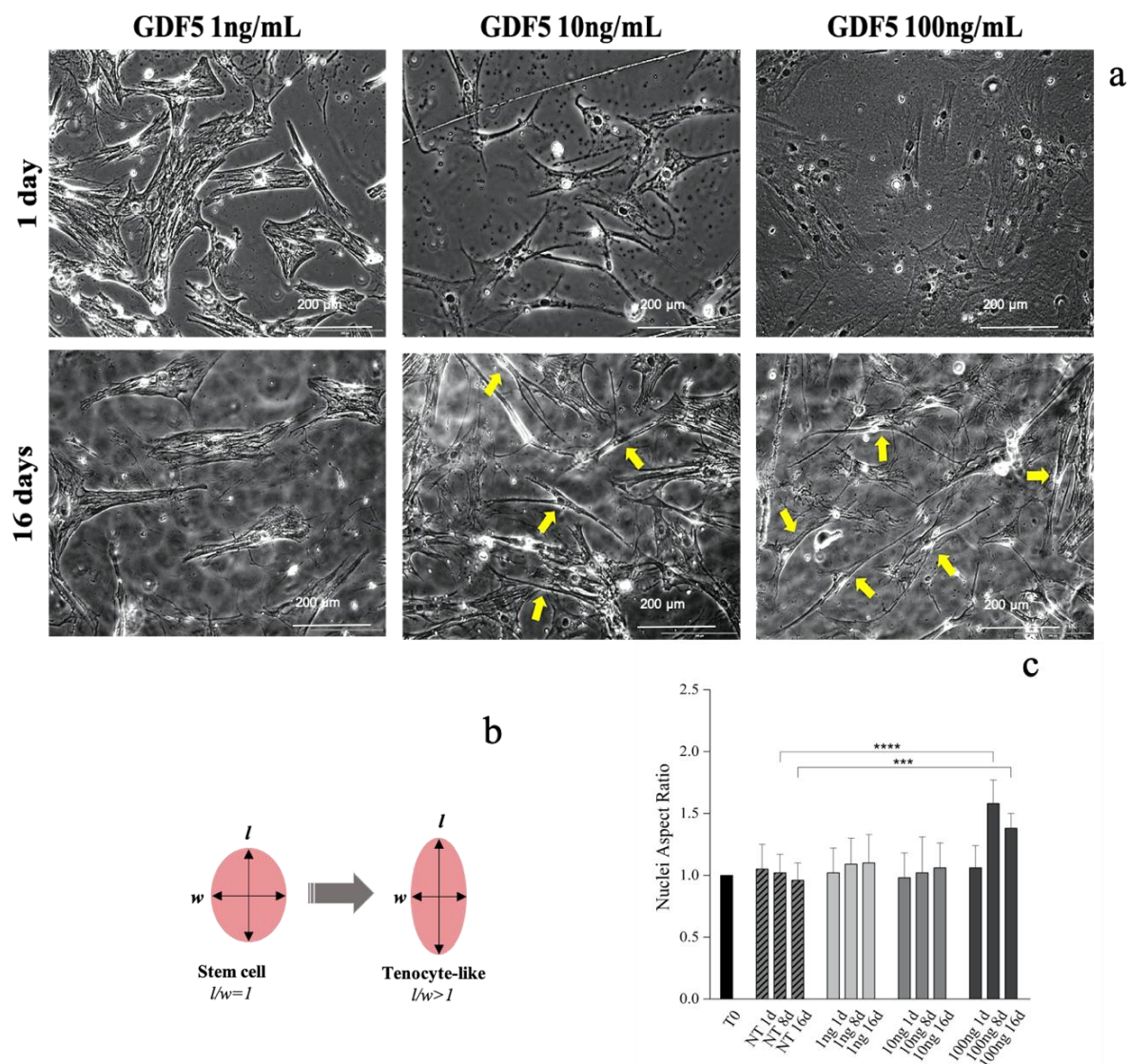


Figure 3. Morphometric analysis of hBM-MSCs with the hGDF-5 concentration-dependent effect.

Brightfield images and shape modification analysis illustrated the hGDF-5 concentration-dependent effect on cells that showed specific alignment and shape modification (fibroblast-like) with an L/W ratio increase at value higher than 1 at Day 16. The average nuclei aspect ratio (length versus width of nucleus) at days 8 and 16 showed statistically significant differences. Statistically significant differences are shown as ***= $p \leq 0.005$; ****= $p \leq 0.001$.

Transcriptional analysis of hBM-MSC supplemented with hGDF-5 (1 ng/mL) revealed upregulation of SCX (1.3-fold), COL1A1 (1.3-fold), COL3A1 (1.5-fold), DCN (1.2-fold) and TNC (1.2-fold) after 8 days of culture. Upregulation was transient in nature and had returned to baseline levels or less by Day 16. An hGDF-5 concentration of 10 ng/mL resulted in a similar transcriptional upregulation pattern as before but that peaked at Day 1 and decreased thereafter (Figure 4b). With 100 ng/mL hGDF-5 supplementation, SCX was overexpressed at day 1 with an upregulation of 1.7-fold and at Day 8 TNMD (12-fold), DCN (1.4-fold), TNC (1.3-fold), COL1A1 (1.3-fold), and COL3A1 (1.2-fold) were observed (Figure 4c). All transcripts were down-regulated thereafter at day 16.

A key correlator with tendon repair and MSC function is immunological responsiveness and modulation. An hGDF5 supplementation of 1 ng/mL with hBM-MSCs upregulated both pro-inflammatory (IL-6: 4-fold; TNF: 4-fold; IL-12A: 6-fold and IL-1 β : 11.5-fold) and anti-inflammatory (IL-10: 3-fold and TGF β 1: 2.5-fold) cytokines, at Day 8, but not thereafter (Figure

4d). Conversely, with 10 ng/mL upregulation of IL-1 β (3-fold), only, was noted at Day 8. (Figure 4e). The highest dose of hGDF-5, 100 ng/mL, was accompanied by significant up-regulation of IL-1 β (11-fold) and IL-10 (10-fold) at Day 8, only (Figure 4f).

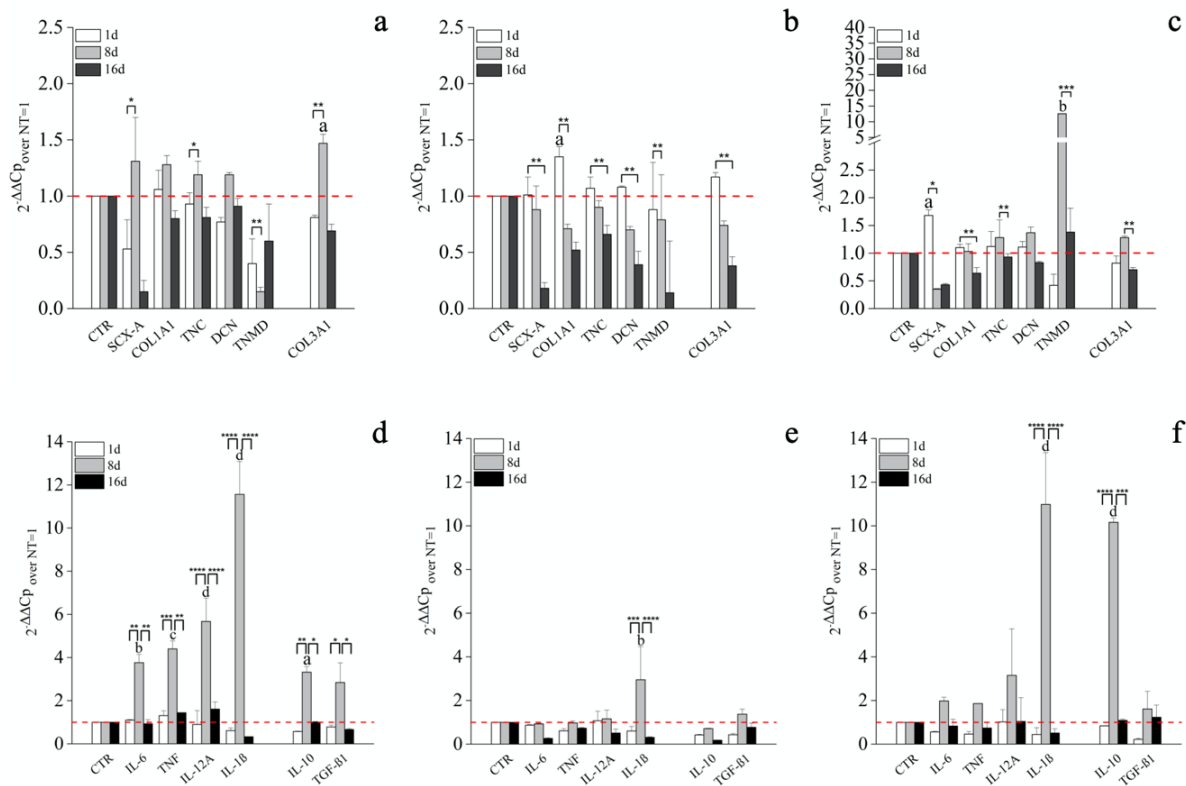


Figure 4. RT-PCR on the expression of tenogenic and cytokine markers by hBM-MSCs. The mRNA levels of tenogenic markers (COL3A1, COL1A1, DCN, SCX-A, TNMD and TNC) and of both pro-inflammatory (IL-6, TNF, IL-12A and IL-1 β) and anti-inflammatory (IL-10 and TGF- β 1) cytokines were monitored; three different concentration of hGDF-5 were tested: (a, d) 1 ng/mL, (b, e) 10 ng/mL and (c, f) 100 ng/mL up to 16 days. Untreated cells for matched time-points selected were used for control purposes. At the lowest concentration tested, mRNA levels of COL3A1, COL1A1, DCN and TNC needed 8 days to be up-regulated (a); increasing the hGDF-5 concentration at 10 ng/mL, the mRNA level for the same genes was up-regulated after just 1 day of treatment (b). Further increasing the hGDF-5 concentration by 100 times (100 ng/mL), mRNA level for SCX-A was again up-regulated after just 1 day of treatment and the expression of TNMD significantly increases of 11 folds after 8 days (c). Moreover, with 1 ng/mL of hGDF-5, both pro-inflammatory and anti-inflammatory cytokines were up-regulated at Day 8 (d); with 10 ng/mL of hGDF-5 only IL-1 β showed a significant up-regulation at Day 8 (e), whereas a better balance of pro-inflammatory and anti-inflammatory cytokines was observed with 100 ng/mL of hGDF-5 at Day 8 (f). Statistically significant differences are shown as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.005$, **** = $p \leq 0.001$; a = $p \leq 0.05$, b = $p < 0.01$, c = $p \leq 0.005$, d = $p \leq 0.001$ compared to NT.

Extending our examination of hGDF5 induction of tenogenic gene expression into hWJ-MSC we noted that cells displayed an aligned phenotype with a fibroblast-like shape, coupled to a L/W ratio value higher than 1, after 7 days of treatment, with a significant increase of 1.8-fold achieved only with 100 ng/mL hGDF-5 supplementation (Figure 6a-c).

Looking at RT-qPCR data, 1 ng/mL was associated with upregulation of SCX-A (2-fold) and COL1A1 (1.3-fold) at Day 3 and DCN (1.5-fold) at Day 7 only (Figure 7a). Similarly, 10 ng/mL had little effect on tenogenic gene expression with the exception of TNC, which increased 1.3-fold at Day 3 (Figure 7b). In contrast to above, and similar to hBM-MSC, 100 ng/mL hGDF-5 induced significant up-regulation of SCX-A (3.8-fold), COL1A1 (3-fold), TNC (2.3-fold), DCN (2.7-fold), and COL3A1 (2.9-fold) at Day 3 (Figure 7c). Evaluation of pro- and anti-inflammatory cytokine expression

following on from hGDF5 supplementation revealed upregulation of TNF and IL-10 (both ~1.5-fold) at Day 3 with 1 ng/mL (Figure 7d). With 10 ng/mL hGDF-5 supplementation we again noted upregulation of TNF (2-fold) and IL-10 (1.2-fold) at Day 3 and noted additional significant increases in IL-1 β levels (1.3-fold) at Day 7 (Figure 7e). Finally, with 100 ng/mL hGDF5 supplementation, we noted significant upregulation of IL-12A (1.5-fold) and, again, IL-10 (2.5-fold) at Day 3 (Figure 7f).

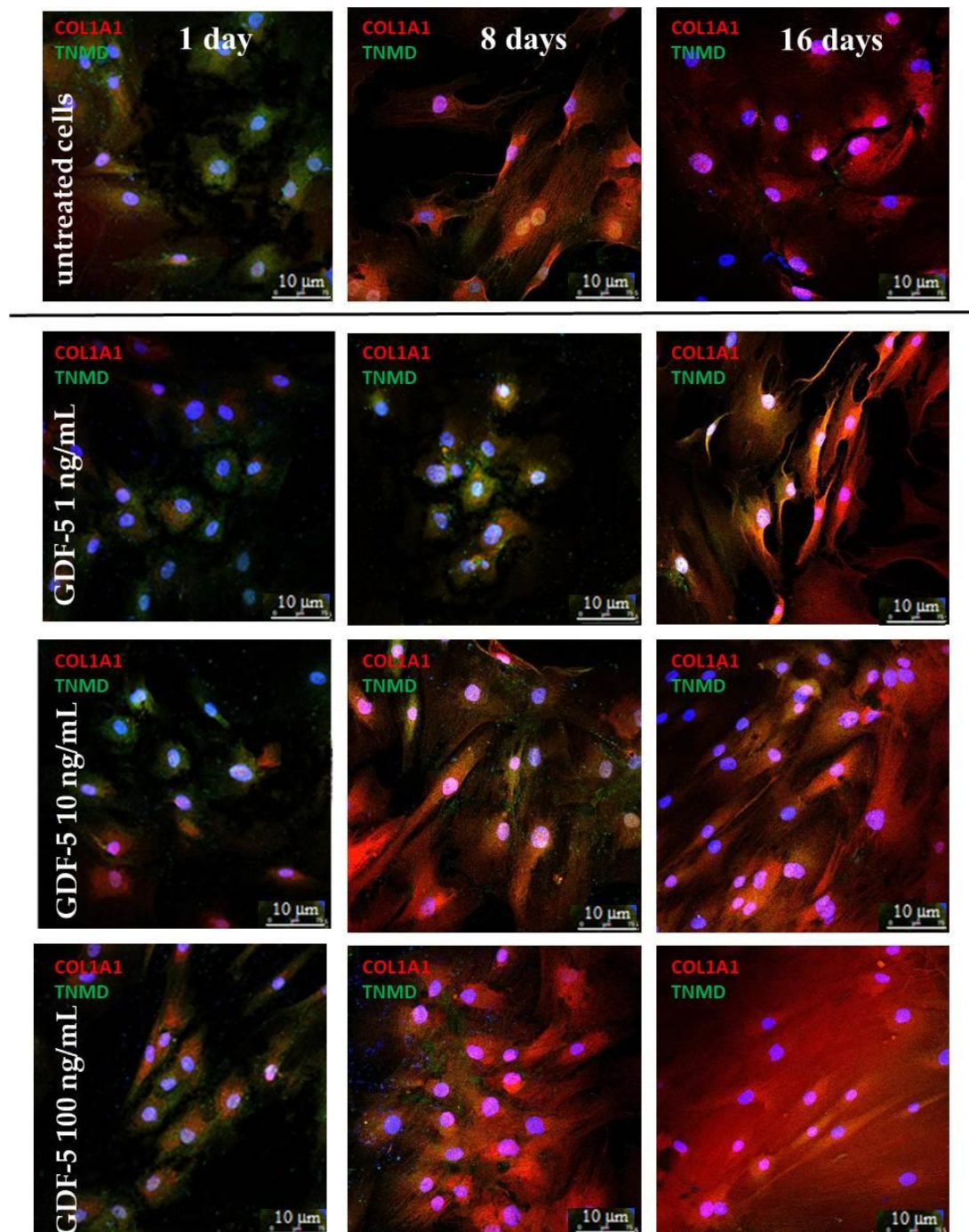


Figure 5. IF images illustrating the effect of hGDF-5 treatment on the expression of type 1 Collagen (COL1A1) and Tenomodulin (TNMD) on hBM-MSCs. Concentration of 1 ng/mL of hGDF-5 did not stimulate significantly the expression of COL1A1 (red staining) which was evident only at day 16. hGDF-5 concentration of 10 ng/mL and 100 ng/mL increased COL1A1 signal at day 8 and 16, even though greater staining was observed when 100 ng/mL were used. TNMD (green staining) was detected only using 100 ng/mL of hGDF-5. At day 8 and 16, cells began to show an aligned pattern and an elongated shape; this behavior was more evident when using 100 ng/mL.

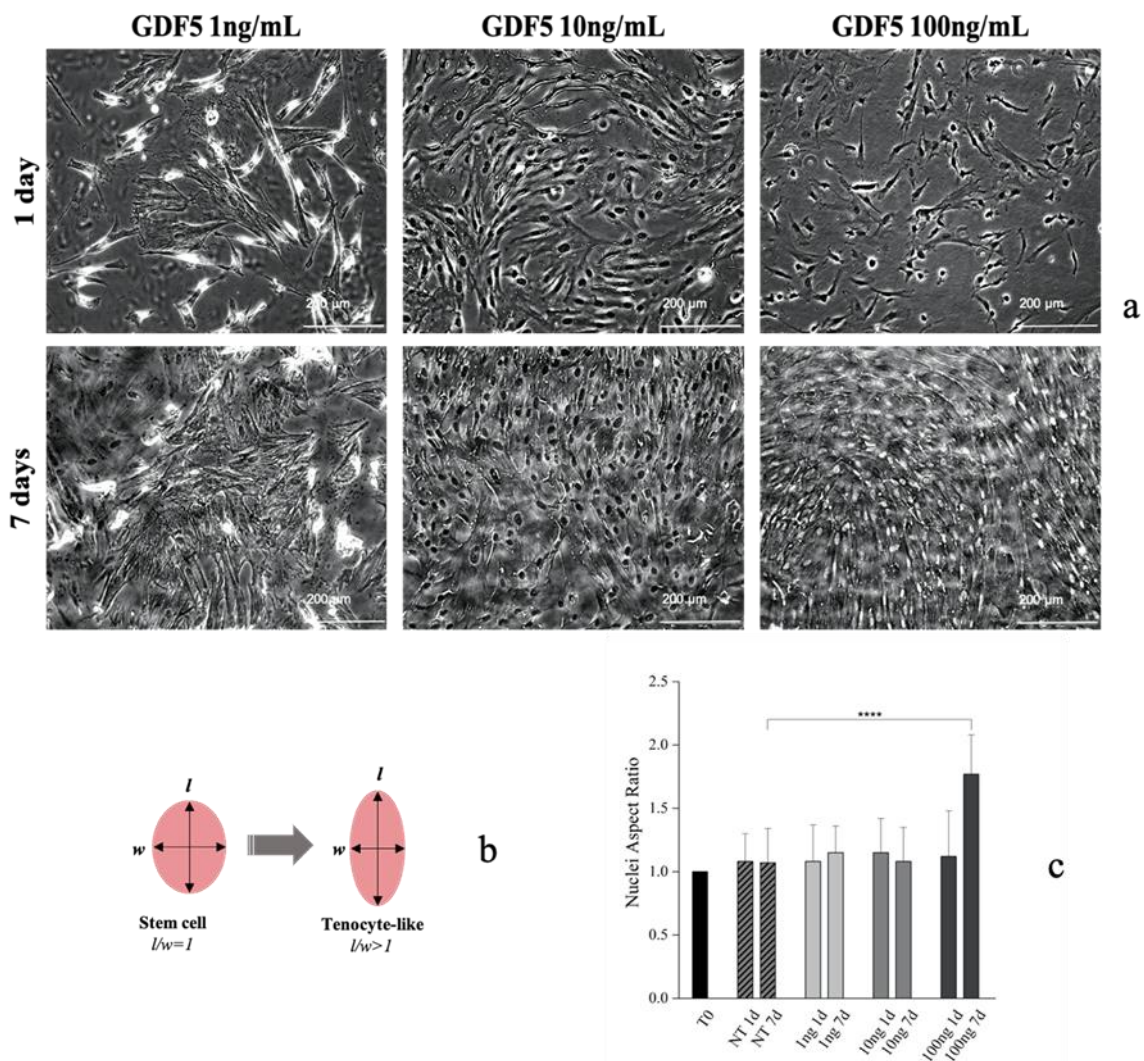


Figure 6. Morphometric analysis of hWJ-MSCs with the hGDF-5 concentration-dependent effect.

Brightfield images and shape modification analysis illustrated the hGDF-5 concentration-dependent effect on cells that showed specific alignment and shape modification (fibroblast-like) with an L/W ratio doubled at Day 7 with 100 ng/mL supplemented with respect to the untreated cells (c).

****= $p \leq 0.001$.

Collagen I and Tenomodulin protein expression were investigated by quantitative immunofluorescence assay across both hBM-MSC and hWJ-MSC populations. Representative images for hBM-MSC (Figure 5), hWJ-MSC (Figure 8 and 9), and their quantification (Figure 10) are shown.

With hBM-MSC, 1 ng/mL of hGDF-5 was associated with no upregulation of either protein. Collagen I expression increased by 1.5-fold and 2-fold after treatment with 10 and 100 ng/mL of hGDF-5, respectively, at day 1 (Figure 10a), and was accompanied by Tenomodulin increases (1.5-fold) after treatment with 10 and 100 ng/mL of hGDF-5 at day 1 (Figure 10b). Similar to hBM-MSCs, untreated hWJ-MSCs showed an increase in basal COL1A1 over time. Moreover, aggregation into 3D spindle-like structures were observed when cells were treated with 1 ng/mL of hGDF-5 while tubular-like 3D structures were observed when 100 ng/mL of growth factor were used after 7 days of treatment. This tubular structure have been already reported by Barboni et al. and may be considered an early organization of cellular 3D structure [61]. Tenomodulin was present within these spindle-shaped and tubular shaped aggregates. Quantification of immunofluorescent hWJ-MSCs images indicated that the COL1A1 signal was significantly increased by 1.5-fold and

2.0-fold after treatment with 100 ng/mL of hGDF-5 at Day 1 and Day 7, respectively (**Figure 10c**). TNMD staining showed a significant increase of 1.5-fold with 100 ng/mL of hGDF-5 at Day 7 (**Figure 10d**).

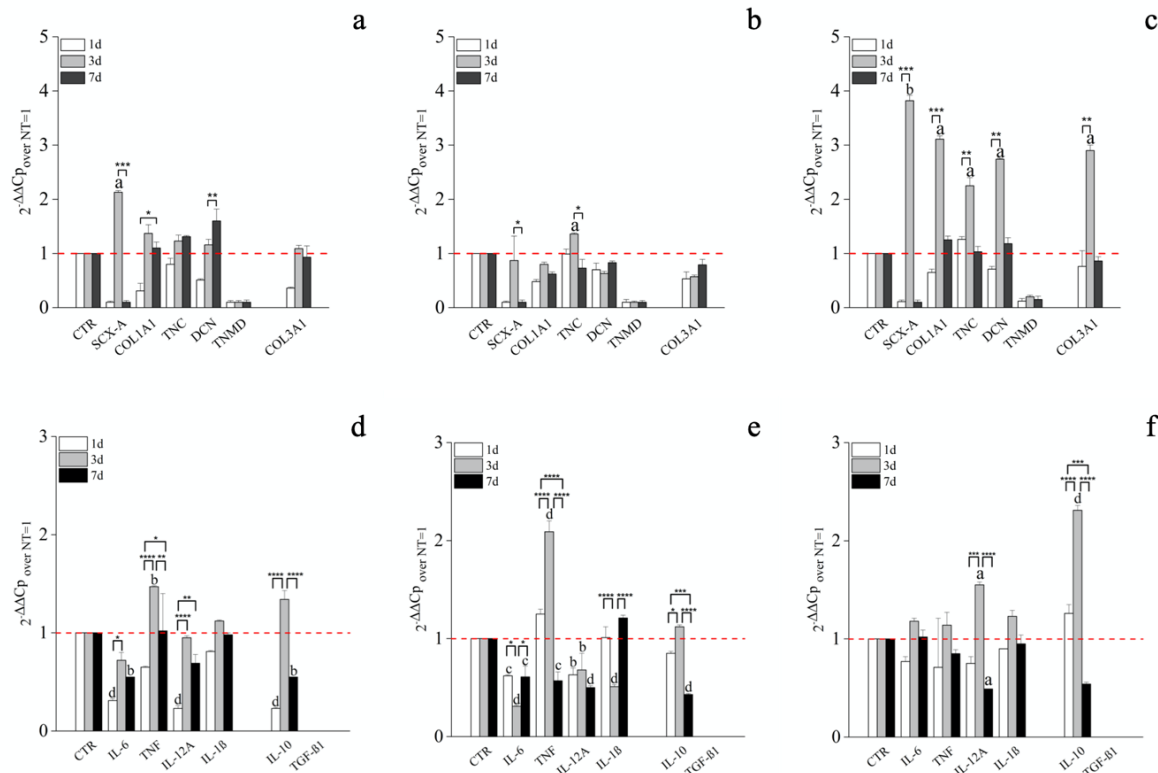


Figure 7. RT-PCR on the expression of tenogenic and cytokine markers by hWJ-MSCs. The mRNA levels of COL3A1, COL1A1, DCN, SCX-A, TNMD and TNC, and of both pro-inflammatory (IL-6, TNF, IL-12A and IL-1 β) and anti-inflammatory (IL-10 and TGF- β 1) cytokines were monitored at hGDF-5 concentrations of 1 ng/mL (**a**, **d**), 10 ng/mL (**b**, **e**) and 100 ng/mL (**c**, **f**) up to 7 days. Untreated cells for matched time-points selected were used for control purposes. Expression of COL1A1, SCX-A and TNC was increased at 1 ng/mL of hGDF-5 at Day 3 (**a**). 10 ng/mL was not the optimal growth factor concentration for the up-regulation of tenogenic markers, except for TNC, which increased (0.3 fold) at day 3 (**b**). Expression of COL3A1, COL1A1, DCN, SCX-A and TNC was significantly increased in cells treated with 100 ng/mL at day 3 (**c**). At the lowest concentration of hGDF-5, the mRNA levels of all the cytokines tested, except TNF and IL-10, was down-regulated (**d**), mRNA levels of TNF increased with 10 ng/mL of hGDF-5 at Day 3 (**e**), whereas, with 100 ng/mL of hGDF-5 hWJ-MSCs showed a slight up-regulation of IL-12A but an interesting and greater up-regulation of IL-10 at Day 3 (**f**). The mRNA for TGF- β 1 was always absent at all the tested concentration of hGDF-5 (**d-f**). Statistically significant differences are shown as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.005$, **** = $p \leq 0.001$; a = $p \leq 0.05$, b = $p < 0.01$, c = $p \leq 0.005$, d = $p \leq 0.001$ compared to NT.

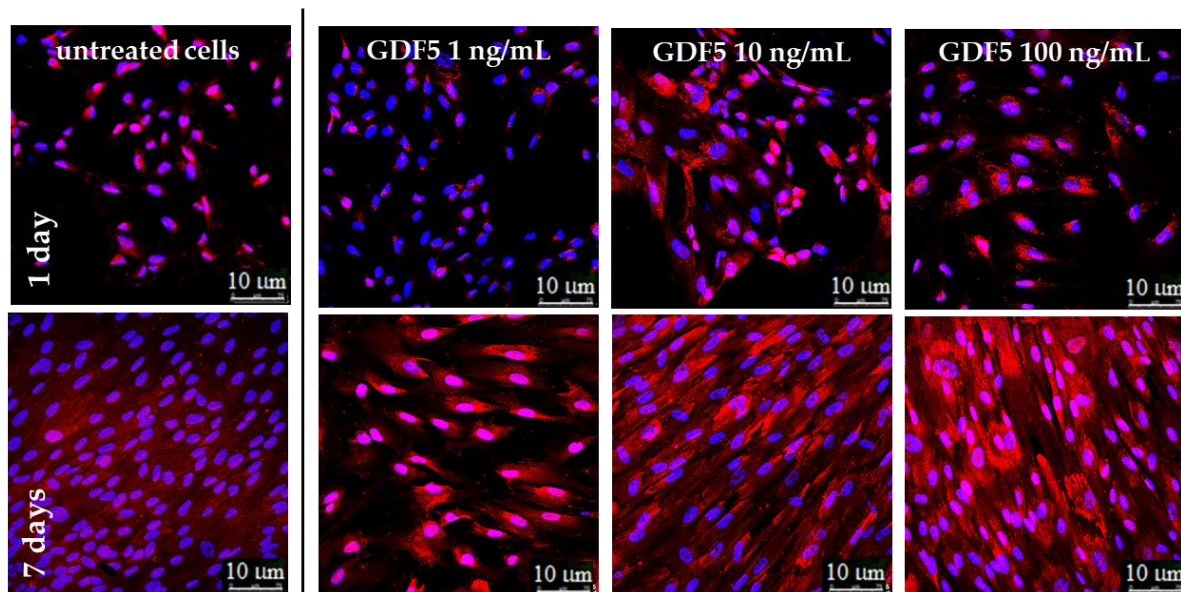


Figure 8. IF assay illustrating the effect of hGDF-5 treatment on the expression of type 1 Collagen (COL1A1) on hWJ-MSCs. hWJ-MSCs showed type 1 collagen expression at day 1. Then, COL1A1 production seemed to increase at day 7 and the amount produced increased following proportionally the incremental hGDF-5 concentration of 1 ng/mL and 10 ng/. An even greater protein content was monitored when hGDF-5 was used at 100 ng/mL at day 7. Cells also showed a great proliferation activity, an evident reshaping in structure that appeared more elongated (fibroblast-like) coupled with an ordinate cells alignment along a given direction. This event was more pronounced at 100 ng/mL.

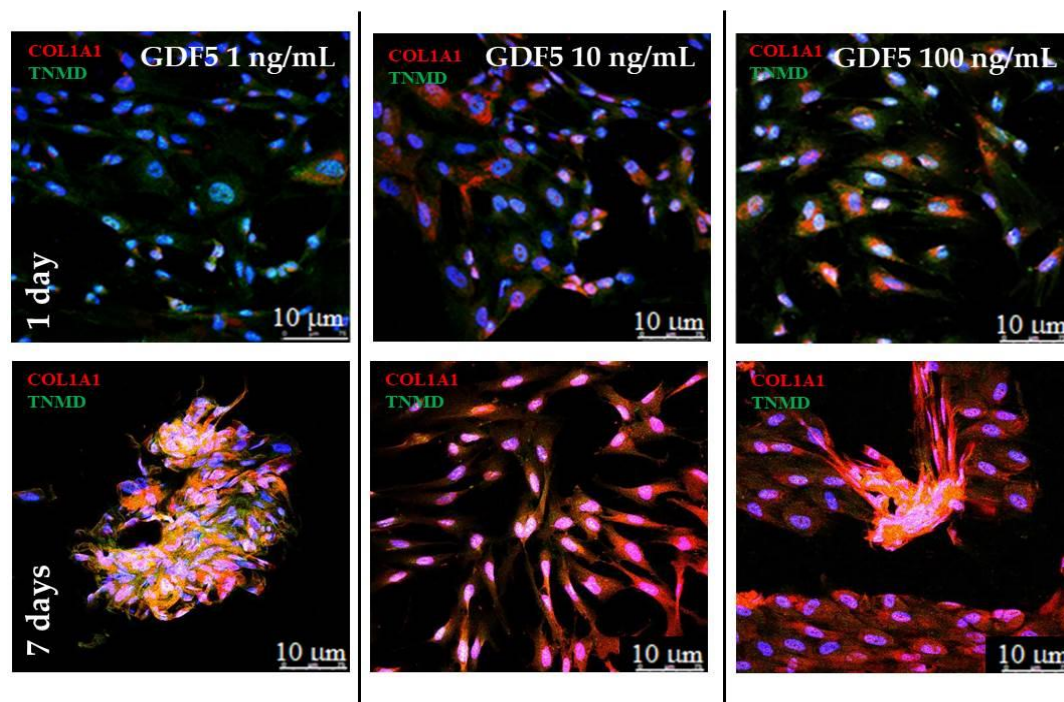


Figure 9. IF illustrating the effect of hGDF-5 treatment on the expression of type 1 Collagen (COL1A1) and Tenomodulin (TNMD) on hWJ-MSCs. After 7 days, hWJ-MSCs aggregated into a spindle shaped system when 1 ng/mL was supplemented; no spindle aggregates were monitored when a concentration of 10 ng/mL was used. Tubular-like 3D aggregates were observed at 100 ng/mL. COL1A1 and TNMD were detected within these 3D aggregates.

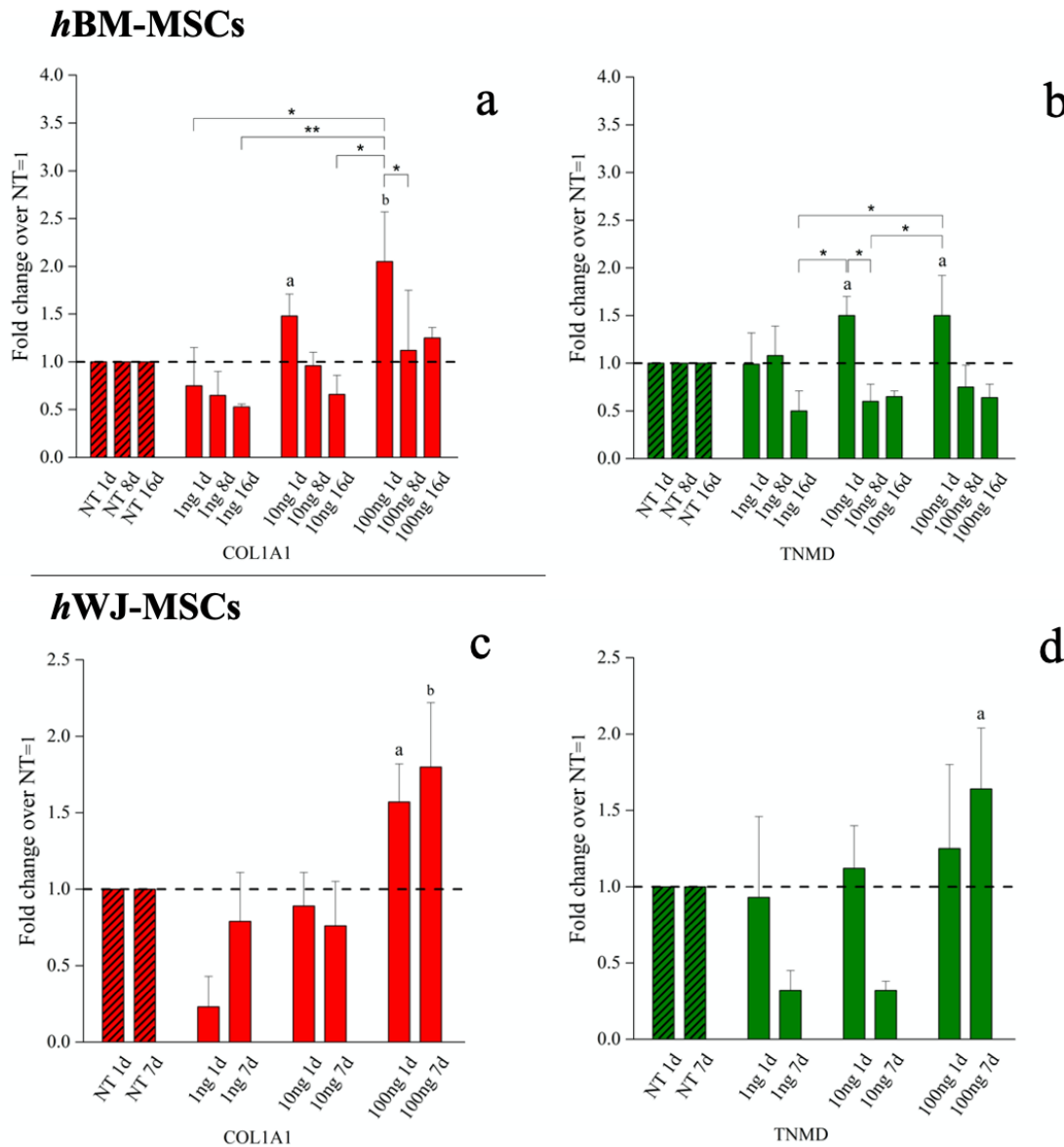


Figure 10. q-IF assays illustrating the exposure relationship of hGDF-5 treatment on the expression of type 1 Collagen (COL1A1) and Tenomodulin (TNMD) on both hBM-MSC and hWJ-MSCs. COL1A1 (stained in red) and TNMD (stained in green) proteins were monitored; untreated cells (NT) are reported for comparison purpose. In the case of hBM, COL1A1 signal increased by 1.5-fold and 2-fold after treatment with 10 ng/mL and 100 ng/mL of hGDF-5, respectively, at day 1 (**a**). TNMD signal increased (1.5-fold) after the treatment with 10 and 100 ng/mL of hGDF-5 at day 1 (**b**). In the case of hWJ-MSCs, the COL1A1 increase is even more evident (2 fold at day 7) compared to the control with treatment at 100 ng/mL (**c**); TNMD also at day 7 with respect to the untreated cells (up to 1.7 fold) (**d**). Statistically significant differences are shown as *= $p \leq 0.05$; **= $p < 0.01$; ***= $p \leq 0.005$; ****= $p \leq 0.001$; a= $p \leq 0.05$, b= $p < 0.01$ compared to NT.

3. Discussion

We hypothesized that hMSCs from bone marrow (hBM-MSCs) supplemented with hGDF-5 would result in upregulation of genes and proteins consistent with tenogenic differentiation, and consequently, synthesize and secrete these into the extracellular environment. We further hypothesized a correlation between hMSCs differentiation and their immunomodulatory activity, through cytokines expression. We utilised hMSCs, isolated from umbilical cord (hWJ-MSCs), to determine their comparability and behavior in the same conditions (three different concentrations of

hGDF-5) and their possible *in vitro* use in tendon tissue engineering. Different doses of hGDF-5 (1 ng/mL, 10 ng/mL and 100 ng/mL) were selected to explore a dose-response effect with regard to tenogenic commitment and cytokine profile behaviors.

hGDF-5 exerted no mitogenic effects on hBM-MSCs enabling us to explore the cells behavior for up to 16 days, acquiring further data with respect to previous studies reported in the literature which described the same cells characterization against a maximum of 10 days [33]. Our data are in strong agreement with previous literature in confirming the dose of 100 ng/mL as optimal for cells commitment toward a tenogenic phenotype. This observation was confirmed by both morphometric analyses, that evidenced nuclei elongation with an increase of 1.4 fold of nuclei length/width ratio, and specific gene over expression at progressively earlier time points when increasing the growth factor dose.

We elected to monitor the gene expression of TNC and SCX because TNC is an ECM glycoprotein considered an early marker of tenogenic differentiation, abundantly expressed in the musculoskeletal tissues during embryogenesis [62], while SCX is a tendon-specific basic helix-loop-helix transcription factor responsible for the transition of MSCs into tendon progenitors [63]. Both TNC and SCX are particularly responsive to mechanical loading [62][39][40], though we did not study the effects of mechanical stimuli in the present study. Nevertheless, these markers were upregulated 1.3-fold after day 8 with 1 ng/mL of hGDF-5, suggesting an activation of the tenogenic commitment pathway after 8 days of treatment. The same activation was earlier and more pronounced when 100 ng/mL were supplemented, with SCX overexpressed 1.7-fold at day 1 and TNC 1.3-fold, at day 8. DCN was monitored because is the small leucine-rich proteoglycan involved in the regulation of fibrillogenesis, and is a fundamental component of the tendon extracellular matrix (ECM), binding to type 1 collagen fibrils [64]. This protein was again not really overexpressed by hBM-MSCs, except at day 8 using 100 ng/mL (1.3-fold); this slight increase can be probably explained because the cells do not reach the complete organization of the ECM in all the experiments considered.

COL1A1 is the major component of tendon tissue (75–85% of the dry mass of tendon), and is the responsible for its mechanical strength [65]. The up-regulation of COL1A1 genes by hBM-MSCs reached 1.5-fold, even when a dose of 100 ng/mL of GDF5 was used in our experimental setting, despite other authors reporting greater gene expression. Tan et al. [33] reported similar results to the ones evidenced in this study. Nevertheless, the expression of TNMD was significantly increased, up to 12-fold, at the concentration of 100 ng/mL of hGDF-5 after 8 days of treatment, suggesting a fairly-good cell commitment versus the specific tendon phenotype. Indeed, TNMD is a type II transmembrane protein, commonly detected in differentiated tenocytes, and is responsible for the organization of the collagen fibers in the late phase of tendon development [2][66]. The expression of this gene was not monitored by the same authors [33], and therefore a comparison is not possible. Furthermore, at 100 ng/mL of hGDF-5, the COL3A1 upregulation was reduced to only 1.3-fold after 8 days; indeed, this gene is basically over expressed by hBM-MSCs, and is the main responsible of fibrotic and scar tissue arrangement [67], and has been reported at the site of rupture of human tendons [65]. Regarding the mRNA levels of pro-inflammatory and anti-inflammatory cytokines, we noticed a strong up-regulation of pro-inflammatory cytokines when the lowest concentration of hGDF-5 was used, above all IL-1 β , which reached a 11.5-fold increase at Day 8. However, the strong up-regulation of IL-1 β was better balanced by IL-10 (10-fold increase) when 100 ng/mL of hGDF-5 were used, suggesting that a differentiation process occurs and it could be accompanied by the immunomodulatory activity of hBM-MSCs in an anti-inflammatory fashion, as reported in literature [57].

The commitment of hBM-MSCs was confirmed by quantitative immunofluorescence assay, which involved the use of Image J soft. Application (NHS). This investigation showed a basal level of collagen type 1 protein (red staining) which was also evident in control cells, as largely documented [68], but a great over expression of this protein was evident when 100 ng/mL were supplemented, while, TNMD (green staining) was evident only at the highest hGDF-5 dose tested. IF observations confirmed that cells showed an ordinate aligned pattern along a specific direction at a hGDF-5 dose of 100 ng/mL at day 16 with evident shape change in the cells, which became elongated, from cells cytoskeleton reorganization versus a more fibroblast-like structure, which is the typical tenocyte

overall appearance. The overall data are in good agreement with the literature, and confirmed that hBM-MSCs are the gold standard to set *in vitro* protocols to study tenogenesis or tendon differentiation and healing.

Taking these indications into account, the same treatment was imparted on hWJ-MSCs which showed several similarities but also some differences. First, given their faster population doubling levels (**Figure 2**) [44], culture longer than 7 days was not possible, even though several and considerable lower seed densities were explored. Nevertheless, a fibroblast-like shape, coupled to a L/W ratio value higher than 1 and increased of 1.8 fold was achieved after only 7 days of treatment when 100 ng/mL of hGDF-5 was supplemented. Since an early expression of SCX-A is reported to be a highly specific marker for tenocyte progenitor cell populations [39] and TNC upregulation intervenes at the very beginning of tendon development [11], we can state that also PCR data were favorable with a clear statistically significant overexpression of SCX-A (3.8-fold), COL1A1 (3-fold), TNC (2.3-fold) and DCN (2.7-fold) observed with 100 ng/mL of hGDF-5 at day 3. However, hWJ-MSCs in this conditions overexpressed also COL3A1 which increased 2.8-fold, compared to the control, at Day 3. The overexpression of COL3A1 was always higher compared to hBM-MSCs. Nevertheless, we have to take into account that an early tenogenic commitment can be manifest by over-expression of SCX-A and COL1A1, an expected outcome of the tenogenic differentiation process, as stated before because SCX-A regulates tendon formation and several other characteristics gene expression [64]. SCX-A regulates also the expression of TNMD [64], but, since TNMD is a marker of mature tenocytes, we suspect that the expression of this gene was not observed in the case of hWJ-MSCs because it would be further up-regulated if our experiments were extended to a longer period of time. However, the early tenogenic commitment of hWJ-MSCs observed with 100 ng/mL at Day 3 was accompanied, similarly to hBM-MSCs, by a modulation of the immune response up-regulating the anti-inflammatory cytokine, such as IL-10 (2.5-fold). Unfortunately, to best of our knowledge, this particular aspect of the behaviour of hWJ-MSCs have never been studied, so the hypothetical correlation between hWJ-MSCs and their immunomodulatory activity needs further investigations.

q-IF data confirmed the commitment of both hBM-MSCs at 100 ng/mL of hGDF-5, with an increase of COL1A1 and TNMD signals at day 16 and 7, respectively (**Figure 10a-b**). In the case of hBM-MSCs, the overall trend of collagen type 1 is in agreement with literature data, suggesting that the initial phases of tenogenic commitment occurred with different time lines in MSCs, though sharing similar characteristics. Indeed, Park et al. indicated the same GDF-5 concentration as the best for ADMSCs differentiation over 12 days of cultivation [36]. The same cell populations of bone marrow MSCs was studied by Tan et al., who described similar results related to an upregulation of the same tenogenic markers, even if related to only 10 days of hGDF-5 treatment at concentration of 100 ng/mL [33]. In the case of hWJ-MSCs, it is difficult to put our result in other context with published data, because no similar study has been reported to date on hWJ-MSCs. However, under these experimental conditions, they seem to respond activating tenogenic commitment events.

4. Materials and Methods

The protocol for the present study and the written informed consent were reviewed and approved by the Institutional Review Board of San Giovanni di Dio e Ruggi D'Aragona Hospital (Review Board prot./SCCE n. 24988, March, 2015).

4.1. hBM-MSCs isolation and harvesting

Human Bone Marrow Mesenchymal Stem Cells (hBM-MSCs) were obtained from bone marrow of male donors (mean age 38). Donors provided written informed consent in accordance with the Declaration of Helsinki to use their filter residual bone marrow aspirate for research purposes. Briefly, total bone marrow aspirate was directly seeded at a concentration of 50,000 total nucleated cells/cm² in T75 plastic flask (BD Falcon, Bedford, MA, USA) in Minimum Essential Medium Alpha (α -MEM; Corning Cellgro, Manassas, VA, USA) supplemented with 1% GlutaGro™ (Corning Cellgro, Manassas, VA, USA), 10% Fetal Bovine Serum (FBS; Corning Cellgro, Manassas, VA, USA), and 1% Penicillin/Streptomycin and incubated at 37°C in 5% CO₂ atmosphere and 95% relative humidity [69]. After 72h, non-adherent cells were removed by media change, and the adherent cells

were further fed twice a week with new media. On day 14, colonies of adherent cells were detached and re-seeded at 4×10^3 cells/cm² in the same culture conditions. Once cultures reached 70–80% confluence, cells were detached using 0.05% trypsin-0.53mM EDTA (Corning Cellgro, Manassas, VA, USA) and washed with Phosphate-buffered saline (PBS) 1X (Corning Cellgro, Manassas, VA, USA), counted using Trypan Blue (Sigma-Aldrich, Milan, IT) and sub-cultured at a concentration of 4×10^3 cells/cm². Flow Cytometry analysis was performed on cell samples obtained at Passage 1.

4.2. hWJ-MSCs isolation and harvesting

Human Wharton's Jelly Mesenchymal Stem Cells (hWJ-MSCs) were isolated from female donors (mean age 28, unrelated to the male donors) who gave written informed consent in accordance with the Declaration of Helsinki to use their umbilical cord for research purposes. hWJ-MSCs were prepared from fresh human umbilical cord obtained during normal spontaneous vaginal delivery. Briefly, umbilical cord sections, approximately 7.5 cm long, were placed in 0.9% NaCl physiological solution supplemented with Ampicillin-Sulbactam 1g + 500mg, stored at 4°C, and processed within 4h of collection. The umbilical cord was cut into 2.5 cm segments, and washed in fresh transport media to remove blood and debris. Each umbilical cord segment was sectioned longitudinally with sterile scissors, and the visible arteries and veins removed. Each piece was transferred to a tissue culture flask 175 cm² (BD Falcon, Bedford, MA, USA) containing α -MEM (Corning Cellgro, Manassas, VA, USA) supplemented with 10% FBS (Corning Cellgro, Manassas, VA, USA), GlutaGro™ (Corning Cellgro, Manassas, VA, USA), and Penicillin-Streptomycin solution. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cell growth was monitored daily with changes of media twice a week. Upon reaching 100% confluence, cells were detached using 0.05% trypsin-0.53mM EDTA (Corning Cellgro, Manassas, VA, USA) and were washed with PBS 1X (Corning Cellgro, Manassas, VA, USA), counted using Trypan Blue (Sigma-Aldrich, St. Louis, Mo, USA), and sub-cultured at a concentration of 4×10^3 cells/cm². For hWJ-MSCs immunophenotype characterisation flow cytometry analysis was performed on cell samples obtained at Passage 1.

4.3. Flow cytometry

hBM-MSCs and hWJ-MSCs were detached and counted. 1×10^5 cells were incubated at RT for 20 minutes with the following directly conjugated mouse-anti human antibodies: CD90 FITC (Beckman Coulter, Fullerton, CA, USA), CD73 APC (Miltenyi Biotec, Gladbach, DE), CD105 PE (Beckman Coulter, XX), CD45 PC7 (Beckman Coulter), HLA class-II FITC (Beckman Coulter, Fullerton, CA, USA), CD14 PC7 (Beckman Coulter, Fullerton, CA, USA), and CD34 PE (Beckman Coulter, Fullerton, CA, USA). The isotype-matched immunoglobulins IgG1 FITC (Beckman Coulter), IgG1 PE (Beckman Coulter, Fullerton, CA, USA), IgG1 APC (Beckman Coulter, Fullerton, CA, USA), and IgG1 PC7 (Beckman Coulter, Fullerton, CA, USA) were used as negative controls under the same conditions. At least 15,000 total events were acquired with a BD FACSVerser flow cytometer (Becton Dickinson, BD, NJ, USA). Further analysis and plots were generated using the BD FACSuite analysis software. Statistics are summarized in **Table 1**.

4.4. hGDF-5 treatment

hBM-MSCs and hWJ-MSCs (passage 3) were seeded on coverslips in 12 well-plates at a concentration of 4×10^3 cells/cm² and 300 cells/cm², respectively. The different seeding densities were to normalize for the differing cell proliferation times where seeding hWJ-MSCs at a density higher than 300 cells/cm² saw them reach 90% confluence in less than 3 days followed by detachment from the flask surface. Once the cell cultures reached 60% confluence, cells were treated with 1 mL of culture media supplemented with three different concentrations of recombinant human GDF-5 (PeproTech; UK): 1 ng/mL, 10 ng/mL, and 100 ng/mL. Cells were fed twice a week with new media and fresh growth factor supplementation. as a consequence of the different proliferation times, hBM-MSCs were treated for 16 days, and hWJ-MSCs for 7 days. Untreated cells for matched time-points were used for control purposes.

4.5. mRNA isolation and gene expression profile

Total mRNA was extracted from hBM-MSCs and hWJ-MSCs using QIAzol® Lysis Reagent (Qiagen, DE), chloroform (Sigma-Aldrich, Milan, IT), and the RNeasy Mini Kit (Qiagen, DE). For each sample, 1 µg of total mRNA was reverse-transcribed using the iScript™ cDNA synthesis kit (Bio-Rad, Milan, IT). Relative gene expression analysis was performed in a LightCycler® 480 Instrument (Roche, IT), using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) with validated primers for Col1A1, Col3A1, DCN, SCX-A, TNMD, TNC, IL-6, TNF, IL-12A, IL-1β, IL-10 and TGF-β1 (Bio-Rad), and following MIQE guidelines [70]. Amplification was performed in a 10 µL final volume, including 2 ng of complementary DNA (cDNA) as template. The specificity of the amplification products was addressed via melting curve analysis. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (reference gene), applying the geNorm method [71] to calculate reference gene stability between the different conditions (calculated with CFX Manager software; $M < 0.5$). Fold changes in gene expression were determined by the $2^{-\Delta\Delta C_p}$ method, and are presented as relative levels versus untreated cells at each time-point explored. All normalizations were obtained using control untreated samples cultivated along the investigated time points.

4.6. Morphometric and proliferation analysis

Nuclei aspect ratio of hBM-MSCs and hWJ-MSCs was determined at different time points in culture by analyzing optical microscope images processed using ImageJ software. Nuclei aspect ratio was determined by measuring and dividing the length by the width of each nucleus; a total of 50-80 nuclei was measured in each condition using different microscope images. Proliferation of hBM-MSCs and hWJ-MSCs was quantified by counting the number of nuclei in each time point and in each condition of treatment using different microscope images.

4.7. Immunofluorescence assay

Cells were fixed with 3.7% formaldehyde for 30 minutes at room temperature (RT) followed by permeabilization with 0.1% Triton X-100 for 5 minutes and blocking with 1% BSA for 1h. For collagen type 1 and tenomodulin staining, cells were incubated overnight at 4°C with a mouse monoclonal anti-collagen type 1 antibody (1:100, Sigma-Aldrich) and a rabbit polyclonal anti-tenomodulin antibody (1:100; Abcam). Following incubation with the primary antibody, cells were incubated for 1h at RT with the DyLight 649 anti-mouse IgG (1:500, BioLegend, CA) antibody and the Alexa Fluor™ 488 goat anti-rabbit IgG (1:500; Thermo Fisher Scientific, USA). Cell nuclei were stained with DAPI solution (1:1000) for 5 minutes. Images acquisition was at 63X magnification using an inverted Leica laser-scanning confocal microscope (TCS SP5; Leica Microsystems) equipped with a plan Apo 63X/1.4 NA oil immersion objective. Image quantification was performed using image analysis software (ImageJ, National Institutes of Health, USA) by measuring the red and green areas where type 1 and tenomodulin, respectively, are expressed [72][73]. Original images were converted from RGB format into a gray scale (16-bit). Then, the average value of pixel intensity (within a range from 0-dark to 255-white) was calculated for the tagged areas. A minimum of 10 image fields (with a comparable number of cells in each image) were used for the image analysis at each time point for each experiment. Data were expressed as fold change relative to untreated cells at matched time-points.

4.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (6.0 for Windows). For phenotypic analysis, data obtained from multiple experiments ($n=3$) are calculated as mean \pm -SD and analyzed for statistical significance using ANOVA test, for independent groups. Differences were considered statistically significant when $p \leq 0.05$ [74].

5. Conclusions

hGDF-5 induces cellular events of tenogenic differentiation that can be time and concentration dependent. The concentration of 100 ng/mL is more effective in this sense, correlating with an anti-inflammatory modulation of the immune cells response. This behaviour was observed on both stem cells, bone marrow and umbilical cord derived. While the proliferation of hBM-MSCs is only slightly promoted during hGDF-5 treatment, the hWJ-MSCs population maintained a high growth profile. After 7 days and at the dose of 100 ng/mL of hGDF-5, hWJ-MSCs manifested the up-regulation of tenogenic markers and showed a L/W ratio >1.

Although the use of autologous stem cells for tendon healing and regeneration has been described [75] and all tissue engineering studies indicated bone marrow stem cells as the gold standard to promote tendon regeneration [76], hWJ-MSCs have never been studied in this sense and may be an interesting option. A better understanding of the complex interactions and pathways of the biomolecules involved in targeted tissue regeneration is necessary to achieve effective therapeutic outcomes for translation into clinic practice. However, our data provide strong evidence that, given their properties, hWJ-MSCs could be potentially used in *in vitro* tendon tissue engineering.

Author Contributions: Maria C. Ciardulli developed the experimental activity and optimized the protocols and methodology for RT-PCR and q-IF; she also was responsible of the paper draft preparation; Luigi Marino isolated all stem cells and characterized them with formal analysis and validated methodology, Erwin P. Lamparelli was involved in RT-PCR experimental activity and data replication, Maurizio Guida provided the umbilical cord tissue and the methodology for hWJ-MSCs isolation, Nicholas R. Forsyth provided contribution in some data supervision and paper editing; Carmine Selleri provided the bone marrow aspirate and the methodology for hBM-MSCs isolation; Giovanna Della Porta was responsible of all experimental data production, curation and supervision, paper writing and editing and of the research project administration; Nicola Maffulli reviewed the manuscript and was responsible of funding acquisition.

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Abbreviations

| | |
|----------|------------------------------------------------------------------|
| hGDF-5 | human Growth Differentiation Factor-5 |
| hMSCs | human Mesenchymal Stem Cells |
| hBM-MSCs | human Bone Marrow Mesenchymal Stem Cells |
| hWJ-MSCs | human Wharton’s Jelly Mesenchymal Stem Cells |
| COL1A1 | type 1 collagen |
| COL3A1 | type 3 collagen |
| DCN | Decorin |
| SCX-A | Scleraxis A |
| TNC | Tenascin-C |
| TNMD | Tenomodulin |
| RT-PCR | Real Time Polymerase Chain Reaction |
| IF | Immunofluorescence |
| q-IF | Quantitative Immunofluorescence assay with ImageJ software (NHS) |

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