Effect of Growth Differentiation Factor 5 on the Proliferation and Tenogenic Differentiation Potential of Human Mesenchymal Stem Cells in vitro

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Key Words
Tendon • Tissue engineering • Cell-based therapy • Orthopedics • Mesenchymal stem cell

Abstract
The use of growth differentiation factor 5 (GDF-5) in damaged tendons has been shown to improve tendon repair. It has been hypothesized that further improvements may be achieved when GDF-5 is used to promote cell proliferation and induce tenogenic differentiation in human bone marrow-derived mesenchymal stem cells (hMSCs). However, the optimal conditions required to produce these effects on hMSCs have not been demonstrated in previous studies. A study to determine cell proliferation and tenogenic differentiation in hMSCs exposed to different concentrations of GDF-5 (0, 5, 25, 50, 100 and 500 ng/ml) was thus conducted. No significant changes were observed in the cell proliferation rate in hMSCs treated at different concentrations of GDF-5. GDF-5 appeared to induce tenogenic differentiation at 100 ng/ml, as reflected by (1) a significant increase in total collagen expression, similar to that of the primary native human tenocyte culture; (2) a significant upregulation in candidate tenogenic marker gene expression, i.e. scleraxis, tenascin-C and type-I collagen; (3) the ratio of type-I collagen to type-III collagen expression was elevated to levels similar to that of human tenocyte cultures, and (4) a significant downregulation of the non-tenogenic marker genes runt-related transcription factor 2 and sex determining region Y (SRY)-box 9.
at day 7 of GDF-5 induction, further excluding hMSC differentiation into other lineages. In conclusion, GDF-5 does not alter the proliferation rates of hMSCs, but, instead, induces an optimal tenogenic differentiation response at 100 ng/ml.

Introduction

Tendon damage resulting from trauma contributes to a large number of soft tissue injury cases reported each year [Butler et al., 2004]. At present, surgical repair of damaged tendon is the only viable option to restore tissue integrity. However, this method does not completely return the damaged tendon to its preinjured state [Butler et al., 2004]. It has been demonstrated that natural tissue healing of tendons appears to be limited due to poor vascularity [Bergljung, 1970]. This in turn results in a higher tendency for the damaged tendon to undergo tissue degeneration [Romeo et al., 1999]. Recent endeavours in tendon tissue engineering have harnessed the potential of cell-based therapy to promote tendon regeneration. It has been hypothesized that the application of cells to damaged sites will produce superior tissue repair, overcoming the limitations of the natural healing processes [Obaid and Connell, 2010]. Mesenchymal stem cells (MSCs) present a great potential for this type of treatment modality due to their ability to proliferate and differentiate into progenitors of different mesenchymal tissues including tenocyte [Lee and Hui, 2006], while avoiding excessive morbidity to the donor site. However, it has been previously reported that transplantation of undifferentiated MSCs may produce complications such as the formation of ectopic bone in situ [Harris et al., 2004]. To overcome such issues, a number of tissue engineering approaches are presently being developed to induce tenogenic differentiation in human MSCs (hMSCs), or to produce controlled differentiation of hMSCs into the desired tenogenic lineage prior to transplantation. An example of such an approach is the use of growth factors [Farng et al., 2008].

The presence of growth factors has been regarded as an important component in the healing of lacerated tendons [Lou et al., 2001]. This has been supported by recent findings demonstrating that several soluble growth factors, e.g. basic fibroblast growth factor [Sahoo et al., 2010], growth differentiation factor 5 (GDF-5) [Farng et al., 2008] and bone morphogenetic protein 12 (BMP-12 or GDF-7) [Wang et al., 2005; Violini et al., 2009], are able to induce tenogenic differentiation in MSCs. Among these, GDF-5, which is a member of the human BMP family, has been identified as a key biological molecule that can accelerate tendon healing [Aspenberg and Forslund, 1999]. However, presently, there are limited studies investigating the isolated effect of GDF-5 on hMSC proliferation and differentiation in vitro. Previous reports examining the effects of GDF-5 on MSCs were limited to studies with immortalized cell lines [Farng et al., 2008] and rodent adipose tissue-derived MSCs [Park et al., 2010]. The effect of GDF-5 on harvested non-immortalized bone marrow-derived hMSCs, which represents the most commonly obtained source of MSCs [Hass et al., 2011], has not been previously described. A critical issue which has not been specifically addressed by previous studies is the establishment of the optimal concentration of GDF-5 that can induce maximal phenotypic expression of the tenogenic hMSC in vitro. This is of particular importance considering that an optimal condition must be attained in order to effectively repair the damaged tendon. Moreover, this knowledge would be of particular interest since it has been suggested that harvested bone marrow-derived hMSCs may respond differently to GDF-5 as compared to the immortalized cell line [Farng et al., 2008] or to MSCs from different sources [Musina et al., 2006]. Furthermore, bone marrow-derived MSCs have been shown to produce higher amounts of collagen as compared to cells originating from other tissues, possibly resulting in superior tissue repair [Van Eijk et al., 2004]. Therefore, this study was conducted to determine the optimal culture condition for hMSC tenogenic differentiation using GDF-5 as an induction factor and to elucidate the effect of GDF-5 on tenogenic hMSC proliferation. A gradient of GDF-5 concentration was tested, considering that progenitor cells can differentiate into various types of mature cells, e.g. osteocytes, chondrocytes and tenocytes, in response to the amount of signal molecules received. We speculate that the hMSCs will differentiate into mature tenocytes in response to a particular concentration of GDF-5 within the range tested, which remains to be established.

To confirm the occurrence of tenogenic differentiation in hMSCs, a few candidate tenogenic marker genes, which include transcription factor scleraxis (SCX) and extracellular matrix (ECM) genes tenasin-C (TNC), type-I collagen (COL-I), type-III collagen (COL-III) and decorin (DCN) were investigated in the present study. These genes were selected in accordance with previous reports indicating that GDF-5 acts as a molecular cue that regulates tenocyte-specific transcription factor, particularly SCX [Schweitzer et al., 2001], through the
Smad/BMP signaling cascade [Bullough et al., 2008], which in turn regulates the expression of COL-I in tendon tissues [Lejard et al., 2007]. In addition, the expression of genes such as TNC, a mechanoresponsive modulator of matrix formation expressed in high tensional loading tissue such as tendons and ligaments, as well as the ratio of COL-I to COL-III have been previously used as indicators of the tenogenic characteristics in tendon tissues and tenocyte cultures [Maffulli et al., 2000; Yao et al., 2006].

In addition, in the current study, non-tenogenic marker genes, i.e. (1) runt-related transcription factor 2 (RUNX2), a transcription factor which is essential for osteoblast differentiation [Komori, 2006, 2010], (2) sex determining region Y (SRY)-box 9 (SOX9), a transcriptional activator for chondrocyte-specific gene such as type-II collagen [Lefebvre et al., 1997; Haller et al., 2011] for chondrocyte differentiation [Bi et al., 2001; Akiyama et al., 2004; Pan et al., 2008], and (3) nucleostemin (NST), a general stem cell marker gene which has been previously reported to be absent in terminally differentiated cells such as tenocytes [Tsai and McKay, 2002; Zhang and Wang, 2010a], were also analyzed to rule out the possibilities that the cells may undergo differentiation into lineage other than tenogenic lineage. These genes have been analyzed in a previous study to confirm the exclusive occurrence of tenogenic differentiation in patella tendon-derived stem cells treated with platelet-rich plasma [Zhang and Wang, 2010b]. To further strengthen the present study design, primary native human tenocyte (hTeno) culture was used as a positive control for comparison with the tenogenic differentiated hMSCs.

**Materials and Methods**

**hMSC Isolation and Culture**

Ethics approval to conduct this study was granted by the University of Malaya Medical Center Ethics Committee (reference No. 602.22). Human bone marrow was harvested from 3 adult donors (table 1) undergoing intramedullary nailing in the University of Malaya Medical Center. Informed consent was obtained from each donor. hMSCs were isolated from bone marrow samples and expanded in vitro following methods that had been previously established [Tan et al., 2011]. A volume of 2 ml of bone marrow was diluted with 2 ml of phosphate-buffered saline (PBS; pH 7.2) and layered onto 3 ml of Ficoll-Paque Premium (GE Healthcare, Sweden) before undergoing gradient centrifugation at 2,200 rpm for 30 min (Eppendorf 5810R).

The mononuclear layer (second top layer) was then collected and washed twice with Dulbecco’s modified Eagle’s medium (DMEM) low glucose (Invitrogen-Gibco, USA) supplemented with antibiotic/antimycotic 1% (v/v; Invitrogen-Gibco). The isolated mononuclear cells were cultured in growth medium [DMEM low glucose supplemented with 10% fetal bovine serum, antibiotic/antimycotic 1% (v/v) and 200 mM GlutaMAX™(™, Invitrogen-Gibco)] and transferred into T75 tissue culture flasks (Nunc™, USA). The medium was changed at day 5 to remove non-adherent cells, and the subsequent medium change was conducted at 3-day intervals.

To determine whether the cells obtained consisted of pure MSCs, various tests including immunohistochemical staining for specific cell surface markers, cell morphological analyses and the ability of the isolated cells to undergo tri-lineage differentiation, i.e. chondrogenic, adipogenic and osteogenic differentiation, were conducted. The methods used are described in our previous publications [Kamarul et al., 2009a, 2009b].

**Primary Native hTeno Isolation and Culture**

Adult human hamstring tendons free of pathology (n = 3) were obtained from donors who underwent ligamentous reconstruction of the knees and arthroplasty of the knee(s) (table 1). Written informed consent was obtained from each donor. The hamstring tendons that were obtained were kept in PBS supplemented with penicillin-streptomycin 1% (v/v; Invitrogen-Gibco) before further processing. The tendon specimens were processed using the methods modified from Zhang and Wang [2010a]. Briefly, the tendons were minced into approximately 1 mm³ in size under a sterile condition. The explants were then transferred into a 15-ml falcon tube with 0.4 mg/ml type I collagenase in PBS (pH 7.2) and incubated at 37°C in a humidified atmosphere of 5% CO₂ for 2 h to allow for the enzymatic digestion process to occur. The specimens were subsequently centrifuged at 1,800 rpm at 15°C for 5 min. The supernatant was then removed and the pellet was washed twice with 10 ml PBS. Following that, the digested explants were cultured in T75 tissue culture flasks (Nunc) with DMEM high glucose (4.5 g/l glucose; Invitrogen-Gibco), supplemented with 10% fetal bovine serum (Invitrogen-Gibco), 100 units/ml penicillin-streptomycin and 200 μM GlutaMAX-1 (Invitrogen-Gibco). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ and supplemented with fresh culture medium at 3-day intervals. Forty-eight hours after culturing, the digested tissues were discarded and the outgrown cells were maintained at 80–90% of confluency for subculture using trypsin digestion. These primary native hTeno cultures (P2 or P3) were used

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69</td>
<td>male</td>
<td>bone marrow from femur</td>
</tr>
<tr>
<td>2</td>
<td>67</td>
<td>female</td>
<td>bone marrow from femur</td>
</tr>
<tr>
<td>3</td>
<td>75</td>
<td>male</td>
<td>bone marrow from femur</td>
</tr>
<tr>
<td>4</td>
<td>19</td>
<td>male</td>
<td>Hamstring tendon</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>male</td>
<td>Hamstring tendon</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>male</td>
<td>Hamstring tendon</td>
</tr>
</tbody>
</table>

**Table 1. Basic demographics and the origin of tissue samples (both for hMSCs and the primary native hTeno cultures) from the donors**

hMSC Tenogenic Differentiation with GDF-5

**Cells Tissues Organs**

3
as positive controls in the subsequent total collagen and gene expression experiments. The cells isolated appeared to have fibroblastic morphology, similar to that reported by Zhang and Wang [2010a] in rabbit tenocyte culture.

AlamarBlue® Cell Proliferation Analysis of hMSC under GDF-5 Induction

Cell proliferation was assessed using the AlamarBlue assay based on the colorimetric quantitative analytical principle. hMSCs (at P2, n = 3) were seeded in the standard 96-well culture plates at a cell density of 10^4 cells/ml and resuspended in 250 μl of culture medium. GDF-5 at various concentration levels (either 0, 5, 25, 50, 100 or 500 ng/ml) were added to the cultures 3 days after seeding. Cells were incubated for an additional period of 2 days before 25 μl of AlamarBlue reagent (Invitrogen-Gibco) was added to the medium. Culture plates were protected from light with aluminium foil. Absorbance readings at 570 and 600 nm were obtained using a spectrophotometer (Epoch; Biotek, USA) at various time points, i.e. at 0, 2, 4, 6, 12 and 24, 36, 48 and 60 h. Untreated hMSCs cultured in MSC growth medium were used as controls. Three independent experiments were performed, each in triplicates in the 96-well plates.

Tenogenic Differentiation and Total Collagen Colormetric Quantification

hMSCs (at P2, n = 3) were seeded in standard 6-well culture plates at a density of 2 × 10^4 cells per well, with serum-free DMEM supplemented with recombinant human GDF-5 at various concentration levels (0, 5, 25, 50, 100 or 500 ng/ml; Abcam, Inc., Cambridge, UK). The hTeno cultures isolated from tendon tissues were seeded in similar densities to that of hMSCs and were used for comparison. These cells were not supplemented with GDF-5. For dose-response analysis, total collagen expressions were measured at 96 h. Based on the results obtained from this experiment, only three concentrations, i.e. 0, 50 and 100 ng/ml of GDF-5, were selected for further analysis which determines the collagen and gene expression levels at different time points (day 4, 7 and 10).

For time response experiments, total collagen assays were conducted at day 4, 7 and 10 in hMSC culture supplemented with 0, 50 and 100 ng/ml of GDF-5. Total soluble collagen in the culture medium was quantified with colorimetric Sircol™ soluble collagen assay (Biscolor, Ireland). Briefly, the cell culture medium was mixed with Sircol dye reagent with vigorous agitation in a 1.5-ml microcentrifuge tube for 30 min. The mixture was then centrifuged for 10 min at 10,000 g to collect the collagen-dye complex at the bottom of the centrifuge tubes. The unbound dye solutions were later removed by draining the tubes. Subsequently, 1 ml of the alkaline reagent was added to each microcentrifuge tube. As the unbound dye dissolved, the absorbance of the samples was measured at 540 nm. The collagen content in the medium was calculated based on the standard curve plot, with COL-I supplied with the kit as the reference sample. In both the dose- and time-response experiments, three independent experiments, each in triplicate, were performed. Data were presented as the mean ± SD. Statistical analysis was analyzed with SPSS (version 17) software. Comparisons of mean values between the different concentrations and various time points were conducted using one-way analysis of variance. Statistical significance was accepted when the p value was <0.05.

Quantitative RT-PCR for GDF-5 Dose-Dependent Relative Gene Expression Analysis

To induce the expression of tenogenic specific phenotypes, hMSCs were cultured in DMEM supplemented with GDF-5 at 0, 50 and 100 ng/ml. After 4 days, the degree of cell differentiation was determined by quantitative RT-PCR (qRT-PCR). This was achieved by measuring SCX, TNC, COL-I, COL-III, DCN and NST genes expressions. Total RNA was extracted from the hMSC cultures, with and without GDF-5 supplementation. In this study, 1 μg of total RNA was reverse-transcribed into cDNA with the transcriptase high-fidelity cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany). qRT-PCR was performed with a Bio-Rad CFX96™ real-time detection system (Bio-Rad Laboratories, Inc., Hercules, Calif., USA) in a final volume of 20 μl with 10 μl iQ™ SYBR® Green Supermix (Bio-Rad Laboratories), 0.6 μl cDNA samples, and 0.2 μM of each primer (for COL-I, COL-III, SCX, TNC, DCN and NST; table 2). The amplification protocol was as follows: an initial denaturation and activation step at 95°C for 30 s followed by 40 cycles of 95°C for 15 s and 61°C for 45 s. A melting curve program was carried out routinely to confirm the presence of a single product (55–95°C with a heating rate of 0.5°C per second and a continuous fluorescence measurement). The annealing temperature at 61°C was derived empirically with temperature gradients. To estimate amplification efficiency, a standard curve was generated for each target molecule via 5-fold serial dilution of a cDNA pool containing the target gene sequences. Data were analyzed with the CFX manager software. A relative quantification method (with corrected PCR efficiency) [Pfaffl, 2001] was performed. All the data were normalized to GAPDH, which was used as our reference gene, after correcting for differences in amplification efficiency (as recommended in the CFX manager package). Data were presented as log2-fold change (±SD) of relative quantification of target mRNA relative to control samples (untreated hMSCs). Student’s t tests were employed to determine the differences between the untreated and GDF-5-treated samples. For all comparisons, the statistical significance was accepted at 95% confidence interval (p < 0.05).

qRT-PCR for GDF-5 Temporal-Dependent Relative Gene Expression Analysis

To determine the tenogenic lineage commitment in hMSCs treated with 100 ng/ml GDF-5, hMSCs were cultured in DMEM supplemented with 100 ng/ml GDF-5 for 7 days. Cells were harvested at day 4 and 7 for total RNA isolation. Total RNA from the hTeno cultures (P2 or P3) was also isolated and used as positive control, whilst the untreated hMSCs cultured in MSC growth medium were used as negative controls. Similar qRT-PCR protocols as described above were used to analyze the expression of SCX, TNC and two non-tenogenic markers: the osteogenic marker RUNX2 and the chondrogenic marker SOX9 in GDF-5-treated and -untreated hMSCs as well as in hTeno. Data were presented as log2-fold change (mean ± SD) of relative quantification of target mRNA relative to negative control samples (untreated hMSCs).
Results

In vitro Characteristics of the Expanded hMSC

Three to 5 days following the plating of bone marrow mononuclear cells onto the plastic surface of the cell culture flasks, discrete fibroblastic cell colonies with a low degree of cellular heterogeneity appeared to develop in the culture. Over time, there was a gradual shift in cell morphology, with an increasing number of fibroblastic cells and a decreasing quantity of the heterogeneous cells (such as polygonal cells) in the culture. Thus, the final homogeneous cell population obtained at the end of P2 was used for the succeeding differentiation assay to minimize the possible variation contributed by the heterogeneous cell populations.

The isolated cells appeared to conform to the characteristics expected of MSCs, i.e. (1) spindle-shaped plastic adherent features; (2) positive markers for CD44, CD105 and CD166 while being devoid of CD34 and CD45, and (3) able to undergo tri-lineage differentiation, namely chondrogenic, osteogenic and adipogenic differentiation (data not shown) [Kamarul et al., 2009a, 2009b].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Size bp</th>
<th>GenBank accession No./reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-AAC ATC ATC CCT GCC TC TAC TG-3' 5'-CTC CTA CGC CTG CTT CAC-3'</td>
<td>196</td>
<td>NM_002046 [Kuo and Tuan, 2008]</td>
</tr>
<tr>
<td>COL-I</td>
<td>5'-CTG ACT GGA AGA GCG GAG AG-3' 5'-TCT GGG CAA TGC TGG GCT GTG TGG G-3'</td>
<td>129</td>
<td>AY633663</td>
</tr>
<tr>
<td>SCX</td>
<td>5'-CAG CGG CAC ACG GCG AAC-3' 5'-CGT TGC CCA GGT GCG AGA TG-3'</td>
<td>165</td>
<td>BK000280 [Kuo and Tuan, 2008]</td>
</tr>
<tr>
<td>NST</td>
<td>5'-ATG ACC TGC CAT AAG CGG TAT-3' 5'-AAG GGA GCA CGT TTT GGA ACT-3'</td>
<td>131</td>
<td>AK315484</td>
</tr>
<tr>
<td>COL-III</td>
<td>5'-CAG CGG TTC TCC AGG CAA GG-3' 5'-CTC CAG TGA TCC CAG CAA TCC C-3'</td>
<td>179</td>
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</tr>
<tr>
<td>TNC</td>
<td>5'-GGG TCC TCA AGA AAG TCA TCC G-3' 5'-CTG ACT CCA GAT CCA CCG AAC-3'</td>
<td>62</td>
<td>NM_002160</td>
</tr>
</tbody>
</table>

hMSC Tenogenic Differentiation with GDF-5

hMSC Differentiation Using GDF-5 Induction

Images of hMSCs in culture with and without GDF-5 supplementation were captured using phase contrast microscopy (fig. 1). There were no significant differences in the gross morphological appearance between these cells, irrespective of the GDF-5 concentration levels used.

Dose and Temporal Effects in hMSC Proliferation under GDF-5 Induction

The effects of different GDF-5 concentrations on the proliferation rate of hMSCs at different time points were reflected by the absorbance readings obtained from the AlamarBlue assay. There was a pattern of an increase in cell proliferation rate at low concentrations of GDF-5 (5 ng/ml) and, in contrast, the cell proliferation rate appeared to be reduced at a high GDF-5 concentration (500 ng/ml). However, the differences in the hMSC proliferation between the cultures treated with or without GDF-5 were not significant (fig. 2), suggesting that GDF-5 did not alter the proliferation rate of the tenogenic hMSCs.
Fig. 1. Morphological appearance of hMSCs in culture medium supplemented with various concentrations of GDF-5, i.e. 0 (a), 5 (b), 25 (c), 50 (d), 100 (e) and 500 ng/ml (f), as well as with hTeno (g). There were no significant differences in gross appearance in the hMSC cultures with or without GDF-5 supplementation. At 100 ng/ml of GDF-5, cells appeared to be having similar unidirectional proliferation and fibroblastic morphology to that of the hTeno culture.
Dose Response of GDF-5 Induced Total Collagen Expression in hMSCs

The results of total collagen assay of the hMSC culture supernatant revealed that at 100 ng/ml, GDF-5 induced a significantly higher total collagen concentration (9.98 ± 1.70 μg/ml; p < 0.05; table 3) as compared to that at concentration levels of 0, 5 and 25 ng/ml (fig. 3a). This expression level was comparable to that observed in the hTeno culture (10.39 ± 2.32 μg/ml). Expression levels of total collagen were significantly lower than the hTeno cultures in all other concentrations of GDF-5. There were no significant differences in the total collagen concentration between the hMSC cultures in all other GDF-5 concentration levels (table 3).

The subsequent time-response experiments showed a significant increase in the total collagen expression in the culture medium from the hMSC cultures supplemented with 100 ng/ml of GDF-5 at day 4, 7 and 10, as compared to the untreated cultures (fig. 3b). A significant increase in total collagen expression was only observed at day 7 onwards in the hMSC cultures treated with 50 ng/ml of GDF-5. This finding demonstrates that 100 ng/ml of GDF-5 is sufficient to induce a tenogenic response from hMSCs as early as day 4, but with the use of 50 ng/ml of GDF-5, a longer period was required.

Relative Gene Expression Analysis of hMSC Tenogenic Differentiation

There were significant differences in the relative gene expression levels for COL-I and COL-III, DCN, SCX, TNC and NST of the hMSCs grown in 0, 50 and 100 ng/ml of GDF-5 at 96 h (fig. 4). At 100 ng/ml of GDF-5, candidate tenogenic markers, COL-I, SCX and TNC, were significantly upregulated (2.31 ± 0.27, 2.30 ± 1.81 and 3.55 ± 0.27 fold increase, respectively; fig. 4).

There was a significantly higher expression of DCN, the genes related to collagen fiber formation (COL-III) and matrix assembly [Zhang et al., 2006], at 96 h as compared to 0 h (p < 0.05). There was a 1.84 ± 0.28

Table 3. Statistical analysis of total collagen expression in hMSCs cultured at different concentrations of GDF-5

<table>
<thead>
<tr>
<th>Concentrations of GDF-5</th>
<th>5 ng/ml</th>
<th>25 ng/ml</th>
<th>50 ng/ml</th>
<th>100 ng/ml</th>
<th>500 ng/ml</th>
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<tbody>
<tr>
<td>0 ng/ml</td>
<td>0.515</td>
<td>0.520</td>
<td>0.153</td>
<td>0.149</td>
<td>0.178</td>
</tr>
<tr>
<td>5 ng/ml</td>
<td>0.978</td>
<td>0.426</td>
<td>0.007**</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>25 ng/ml</td>
<td>0.157</td>
<td>0.035*</td>
<td>0.007**</td>
<td>0.183</td>
<td></td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>0.153</td>
<td>0.178</td>
<td>0.921</td>
<td>0.149</td>
<td></td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>0.149</td>
<td>0.921</td>
<td>0.149</td>
<td>0.921</td>
<td></td>
</tr>
</tbody>
</table>

Data given are p values. Summary of the least significant difference analysis with Bonferroni adjustment for multiple pairwise comparisons of the mean total collagen differences in the culture medium of hMSCs supplemented with different amounts of GDF-5. The p values are presented at 95% confidence intervals and the significant values are denoted with an asterisk. * p < 0.05; ** p < 0.01.
Fig. 3. Total collagen content analysis for hMSCs cultured with different concentrations of GDF-5. 

a) Dose-response analysis at 96 h following GDF-5 supplementation. The total collagen expression in hMSCs was significantly increased at 100 ng/ml of GDF-5 (* p < 0.05).

b) Time-response analysis at three concentration levels of GDF-5, i.e. 0, 50 and 100 ng/ml. In comparison to hMSCs without GDF-5 induction, the total collagen expression was significantly elevated (* p < 0.05) in hMSCs at 100 ng/ml of GDF-5 on day 4 onwards, whilst significant increase (* p < 0.05) at 50 ng/ml was only observed at day 7 onwards.

Fig. 4. Gene expression analysis of candidate tenogenic markers in hMSCs cultured with GDF-5 at different concentrations (0, 50 and 100 ng/ml). There was a significant upregulation of gene expression of candidate tenogenic marker genes COL-I, SCX, TNC and COL-III at 100 ng/ml of GDF-5. * p < 0.05; ** p < 0.01.
fold increase in the COL-III expression at 50 ng/ml and a 2.56 ± 0.41 fold increase at 100 ng/ml at 96 h. Although the COL-III expressions were not significantly different in hMSCs cultured with GDF-5 as compared to those without GDF-5, the ratio of COL-I to COL-III appeared to be increased (online suppl. table, www.karger.com/doi/10.1159/000335693). For DCN expression, there was a 4.47 ± 0.41 fold increase at 50 ng/ml and a 4.42 ± 0.57 fold increase at 100 ng/ml at 96 h. No significant difference in NST gene expression levels was observed in all groups, demonstrating that despite undergoing tenogenic differentiation, cells maintained their original MSC gene expression.

Having established significant increment in the candidate tenogenic marker gene expression at 100 ng/ml of GDF-5, we further analyzed the temporal effect of GDF-5 at that concentration level on hMSC differentiation. In the temporal analysis, the candidate tenogenic marker genes SCX and TNC were upregulated on day 4 after the GDF-5 treatment (fig. 5a, b). However, on day 7, only SCX was persistently upregulated, whereas the expression of TNC was reduced (p < 0.01). The expression of non-tenogenic marker genes RUNX2 and SOX9 was significantly downregulated (p < 0.01) on day 7 (fig. 5c, d). These findings suggested that hMSCs were undergoing tenogenic differentiation as early as day 4 following GDF-5 induction at 100 ng/ml.

Discussion

The present study demonstrates that 100 ng/ml of GDF-5 provided the optimal concentration required to induce tenogenic differentiation of bone marrow-derived hMSCs. This deduction was based on the following findings: (1) total collagen was significantly higher in cultures with GDF-5 concentrations of ≥50 ng/ml; (2) however, only the concentration of 100 ng/ml in hMSC cultures produced similar levels of total collagen to that of the hTeno cultures. hMSC cultures with GDF-5 concentrations >100 ng/ml produced significantly less total collagen than the hTeno cultures; (3) within the range of GDF-5 concentrations tested, only concentrations of 100 ng/ml produced significantly higher amounts of collagen as early as day 4; (4) COL-I, SCX and TNC genes in the hMSCs were only upregulated at this concentration; (5) the ratio of COL-I to COL-III was highest in hMSC cultures supplemented with 100 ng/ml of GDF-5, and (6) non-tenogenic genes, such as RUNX2 and SOX9, were significantly reduced on day 7 of GDF-5 induction. These findings appear to be supported by a previous study [Park et al., 2010], demonstrating that at 100 ng/ml, there was a significant increase in protein expression of tenomodulin, TNC, Smad-8 and matrix metalloproteinase-13, compared to that of ≤10 ng/ml. At higher concentrations, i.e. 1,000 ng/ml, these genes were downregulated instead [Park et al., 2010]. Although our study did not utilize such high concentrations, at concentrations of 500 ng/ml, the total collagen concentration from the hMSC cultures ap-
peered to be significantly lower than that of the hTeno cultures and hMSC cultures at a concentration of 100 ng/ml. This precluded the need for further experiments beyond the concentration level of 500 ng/ml. It is unclear as to why there was no linear pattern of dose-response relationship in this study. It can be speculated that at higher concentrations of GDF-5, the antagonistic effects of GDF-5 may have resulted in a decrease in collagen production, as observed in other studies using other growth factors, for example, transforming growth factor-β [Ghosh et al., 2001]. However, this remains speculative and needs to be proven in a separate study.

It is interesting to note that GDF-5 did not appear to influence hMSC proliferation in the present study, which is in contrast to a previous report involving the rodent adipose tissue-derived MSC primary culture [Park et al., 2010]. In addition, in a separate study, the rodent adipose tissue-derived MSCs have been reported to produce faster proliferation than those derived from the bone marrow [Saka et al., 2011]. Nevertheless, the data presented in this study appear to be consistent with a study which uses murine bone marrow-derived stromal immortalized cell lines [Fargn et al., 2008]. Therefore, it appears that the tissue of origin of the MSCs may be a predetermining factor affecting cell proliferation in the presence of GDF-5. However, this needs to be confirmed by a more robust study design. In addition to tissue of origin, other factors such as species [Reilly et al., 2007] and the amount of serum used for cell culture [Saka et al., 2011] may also play an important role in determining the response of bone marrow-derived hMSCs to GDF-5. The effects of GDF-5 on MSC proliferation have not been previously explained by a specific cellular mechanistic pathway which may be involved following GDF-5 receptor stimulation. However, there are several possibilities that could be considered. GDF-5 has been shown to bind specifically to three different receptors: serine/threonine kinase receptor type 1 (BMPR1b) or serine/threonine kinase receptor type 2 which could be either BMPR2 or ACTR2a [Nishitoh et al., 1996]. The activation of any one of these receptors may induce the expression of ID1 and ID3 via the Smad-dependent and mitogen-activated protein kinase-independent pathway in the regulation of transcription for cell cycle or cell proliferation control [Chen et al., 2006]. This could be expected considering that mitogen-activated protein kinase is an important signaling pathway that regulates cell proliferation [Zhang and Liu, 2002].

The changes in the candidate tenogenic marker genes (such as SCX, TNC, COL-I and COL-III) as observed in our study strongly suggest that the expression of the main proteins produced by tenocytes, i.e. COL-I, COL-III, TNC and DCN, is also present in the tenogenic differentiated hMSCs. It is plausible that the process of tenogenic differentiation induced by GDF-5 at 100 ng/ml in the present study involves a similar Smad/BMP signal transduction mechanism to that reported previously [Nishitoh et al., 1996]. However, this needs to be substantiated by a more comprehensive study.

The increase in SCX and COL-I is an expected outcome of the tenogenic MSC differentiation process. However, it is interesting to note that DCN expression was not upregulated during tenogenic differentiation, which may have been related to the monolayer conditions used in this experiment [Yao et al., 2006]. Culture conditions play a critical role in modulating the synthesis, assembly and organization of ECM components. In a 3D environment, DCN is also able to modulate the BMP/transforming growth factor-β pathway through interaction with lipo-protein receptor-related protein [Cabello-Verrugio and Brandan, 2007] and regulates matrix organization and mechanical characteristics of the 3D collagen matrices [Ferdous et al., 2007]. Nonetheless, this may not be the case in the present study, as previous studies have shown that an increase in DCN expression in monolayer rodent MSC cultures is not unexpected [Park et al., 2010].

During the course of tenogenic differentiation, the expression of TNC can be expected to be persistently upregulated at a later time point, potentially reaching a level similar to that observed in the hTeno cultures. In this study, the TNC expression in hMSCs at day 7 of GDF-5 induction appeared to be reduced (fig. 5). However, the temporal expression profile observed in this study was similar to that observed in a rodent MSC model, which demonstrated downregulation of TNC on day 6 upon GDF-5 induction which eventually increased on day 9 and day 12 [Park et al., 2010]. Since TNC is a late tenogenic marker, we suspect that the expression of this gene would be upregulated in the hMSCs if our experiment were extended to a longer period of time, as observed in the previous rodent MSC model [Park et al., 2010]. The increased expression of this gene at a late phase of tendon development has recently been documented in a study comparing the TNC expression in tendon from the embryonic period to that observed at day 14 postnatally [Liu et al., 2012]. The observation from our temporal experiment implicates that it may have been appropriate to utilize the GDF-5-induced hMSCs collected at day 4 for further clinical application, in view of the fact that the hMSCs have already been differentiated into tenocytes, as indicated by the significant upregulation of the TNC.
gene expression at this time point. However, a more rigorous study needs to be conducted in order to confirm this.

In this study, although no significant differences were observed in COL-III gene expression levels in hMSCs induced with GDF-5, the ratio of COL-I and COL-III was highest in hMSC cultures supplemented with 100 ng/ml of GDF-5 (online suppl. table), suggesting that at this concentration, hMSCs underwent optimal differentiation along the tenogenic lineage. In the current study, there was an apparent increase in the total collagen expression in the hMSC cultures at 100 ng/ml of GDF-5 (fig. 3), with no significant increase in COL-III gene expression (fig. 4). This suggests that the increase in total collagen expression observed in our study is likely mainly due to an increase in COL-I, but the possibility of the presence of other collagens, e.g. collagen type II, cannot be ruled out completely. However, this would be unlikely considering that other studies have shown that the expressions of these proteins are usually of minimal quantity [Altman et al., 2002].

An interesting finding from the present study is the presence of persistent expression of NST in tenogenic differentiation of hMSCs. The NST gene is a highly specific gene found in MSCs that may also be present in embryonic stem cells and various primitive cells found in the bone marrow. However, this gene is absent in differentiated lineage committed cells found in most adult tissues [Tsai and McKay, 2002]. It has been previously observed that NST expression will decrease rapidly as soon as MSCs (or tendon stem cells) undergo tenogenic transformation [Zhang and Wang, 2010b]. However, this was not observed in the present study. There are at least 2 possible explanations for this contrasting result: (1) the duration of the experiment in the present study may be too short to observe any reduction in the NST gene expression. Should our experiment be extended to a longer period, we suspect that a decrease in NST gene expression may have been observed since the NST+ MSCs may have been attenuated when mixed with the increasing population of the NST− mature tenocytes. (2) The transformation of MSCs from their undifferentiated progenitor origins to tenogenic committed cells in the presence of GDF-5 may be a complex process. In normal circumstances, under GDF-5 induction, hMSCs would be expected to differentiate into tenogenic MSCs, which would subsequently undergo symmetrical cell division and mature into tenocytes (fig. 6a). However, an alternative, more complex, cell differentiation process may have occurred. In this condition, it is plausible that hMSCs might first transform into tendon stem cells, which would undergo asymmetrical cell division resulting in two different cell populations, i.e. tendon stem cells and mature tenocytes. The persistence of tendon stem cells that possess self-renewable capability would therefore retain (or increase) the NST gene expression throughout the differentiation process (fig. 6b). In both scenarios, it is not known whether the tenogenic differentiation is still reversible, such that the tenogenic hMSCs might regress to become undifferentiated hMSCs again. This speculation needs to be further substantiated by a more comprehensive study, for example, using flow cytometry quantification analysis.

Although several limitations were identified within the present study, these were unfortunately unavoidable. Firstly, the use of total collagen assay to evaluate the tenogenic differentiation of hMSCs may not have been the best method of assessment considering that many cells of mesenchymal origin produce an abundance of collagen extracellularly. The assessment of a more specific collagen, for example, COL-I and COL-III, may have been a more reasonable approach considering that these proteins are specific to tenocyte expression. However, the concentrations of these collagen types may not appear in sufficient quantities to be detected by conventional spectrophotometry analyses such as that utilized in the present study. Hence, gene expression analysis, which is a more specific and sensitive tool of assessment, was advocated in the present study. It should be noted that gene expression analysis has its inherent limitation considering the fact that measurements are made at the transcription level rather than reflecting the true level of the ECM protein that is synthesized. The decision to use total collagen assay may have been the most appropriate option in this study, since this approach has also been used in other study to evaluate the tenogenic differentiation of the fibroblasts [Sahoo et al., 2010]. In other studies, changes in total collagen also appear to be correlated to the changes in the conventional hydroxyproline assay, which is an indicator of the amount of tendon-specific collagen [Taskiran et al., 1999]. Secondly, although the data presented here are novel in view of the absence of previous data on primary human bone marrow-derived MSCs undergoing tenogenic differentiation under GDF-5 induction, it would also be useful to carry out direct comparisons between the hMSC derived from the human bone marrow with that of the MSC lines or the primary MSC culture from animal models to further explore the potential influence of tissue of origin on the effect of GDF-5 on the cells. Lastly, one of the commonly used tenogenic marker genes, tenomodulin [Docheva et al., 2005], was not analyzed in this study. Tenomodulin is a type II transmembrane glycoprotein that has been reported to be pre-
dominantly expressed in tendons and ligaments. In this study, although we did not include tenomodulin in our analysis, we have analyzed the expression of SCX which is also a tenogenic marker that positively regulates tenomodulin expression in a tendon lineage-dependent manner [Shukunami et al., 2006]. In addition, the expression of tenomodulin has previously been detected in other tissues such as cartilage, bone marrow and fat tissue [Jelinsky et al., 2010]. Considering that our hMSC primary culture was derived from bone marrow, the analysis of this gene may not have been as relevant as it would have been when using MSCs derived from other tissues.

In conclusion, this study has shown that the use of GDF-5 induces tenogenic differentiation in hMSCs without significant alteration in the cell proliferation rates. It appears that GDF-5 at a concentration of 100 ng/ml provides the most optimal cell phenotypic response, which includes an augmented level of total collagen and tenogenic marker gene expression, as well as a reduced non-tenogenic marker gene expression, similar to that observed in hTeno cultures.

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Disclosure Statement

The authors indicate no potential conflicts of interest.
References


