ENHANCING INFLAMMATORY AND CHEMOTACTIC SIGNALS TO REGULATE BONE REGENERATION

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Abstract

Stem cells have become the fundamental element in regenerative medicine due to their inherent potential to differentiate into various cell types, and the ability to produce various bioactive molecules, including growth factors, cytokines and extracellular matrix molecules. *In vivo*, the secretion of tropic factors is modulated by chemotactic and inflammatory factors. In this study, we analysed the influence of a 2 h stimulation of mesenchymal stem cells (MSCs) with interleukin-1β (IL1β), granulocyte-colony stimulating factor (GCSF), stromal cell-derived factor 1 (SDF1) and stem cell factor (SCF). Our results demonstrated that this short stimulation exerts pronounced effects on the expression of multiple cytokine genes and proteins in MSC cells 48 and 72 h later. IL1β strongly promoted the secretion of a wide range of proteins with chemotactic, proinflammatory and angiogenic properties, whereas SCF regulated the expression of proteins involved in proliferation, chondrogenesis and ECM regulation. This demonstrates that the changes in secretome can be directed towards a desired final functional outcome by selection of the most appropriate cytokine. Moreover, the expression pattern of Wnt signalling pathway components suggested the differential regulation of this pathway by IL1β and SCF. Altogether, the robust paracrine action of MSCs can be achieved within a just 2 h treatment, which would be feasible within the operating theatre during a single surgical procedure. These results suggest that integrating inflammatory modulation in bone tissue engineering, by modifying the MSC secretome by way of a short stimulus, would provide a more targeted approach than administering unmodified MSCs alone.

Keywords: Bone repair, stem cells, cytokines, inflammation, tissue engineering.

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Introduction

Various strategies have been developed and applied to aid the regeneration process of the injured tissue. These include separate or combined implantation of specific cell types, biomaterials and factors. Cell therapy alone, or in combination with supportive biomaterials and factors, is of particular interest for clinical application to treat a wide array of acute or chronic conditions due to the inherent potential of stem cells to differentiate into various cell types (Pittenger *et al.*, 1999). Currently, more than 4,500 ongoing clinical trials have been registered worldwide (*www.clinicaltrail.gov*) using stem cells, and approximately 10 % of these use MSCs. Specifically, much attention and hope is invested into single surgical procedure cell therapies. This strategy integrates the autologous cell harvesting, processing and re-implantation into the injured site within the operation theatre. Intraoperative autologous stem cell therapy brings advantages regarding the usage of patient's own cells, thus avoiding the activation of an immune response, extensive *in vitro* cell expansion and manipulation, requirements for Good Manufacture Practice facilities, a secondary invasive procedure and potential contamination (Coelho *et al.*, 2012).

In addition to their direct contribution to tissue regeneration, as a consequence of differentiation into specific phenotype, implanted cells aid regeneration by producing various bioactive molecules, including growth factors, cytokines and extracellular matrix molecules (Tsiridis *et al.*, 2007; Zhukareva *et al.*, 2010; Hsiao *et al.*, 2012). These secreted factors act in an auto- and paracrine manner regulating cell phenotype. The importance of paracrine actions in the host tissue have been studied extensively in repair and regeneration of the central nervous system (Drago *et al.*, 2013), heart (Gnecchi *et al.*, 2006; Hwang *et al.*, 2012) and skin (Schlosser *et al.*, 2012). The indirect action of progenitor cells implanted within the bone-healing environment is less understood; nevertheless, the importance of various factors secreted by cells present in fracture milieu is known to have great importance on bone regeneration.

The importance of inflammation occurring after bone fracture has been demonstrated directly by the increased levels of various factors at the molecular and protein level, as well as indirectly by removing the fracture haematoma (Kolar *et al.*, 2011). Combating inflammation and, as consequence, the wide spectrum of factors secreted by immune and progenitor cells present at the fracture site has also been shown to have a role (Simon *et al.*, 2002). The local increased levels of mRNA expression of interleukin 8 (IL8), vascular endothelial growth factor (VEGF) together

with interleukin 6 (IL6), CXC chemokine receptor type 4 (CXCR4) and secreted phosphoprotein 1 (SPP1) in fracture haematoma indicate their importance for chemotaxis, angiogenesis and osteogenesis (Kolar *et al.*, 2011).

The removal of the haematoma, together with cells contained within, or combating inflammation with non-steroid anti-inflammatory drugs, prolonged the time of fracture healing compared to fracture with haematoma (Simon *et al.*, 2002; Kolar *et al.*, 2011; Mountziaris *et al.*, 2011).

In order to increase the production of specific factors by cells strategies such as genetic engineering and preconditioning of cells have been applied. Since the secretion of tropic factors is modulated *in vivo* by cytokines, their use could provide a strategy to enhance the paracrine actions of cells prior to their implantation. This could enhance their function during the critical early time points. Driven by this hypothesis we selected four factors which are players in the early stages of the bone healing cascade, including interleukin 1β (IL1β), granulocyte-colony stimulating factor (GCSF), stromal cell-derived factor 1 (SDF1) and stem cell factor (SCF), to determine the cytokine profile of mesenchymal stem cells (MSCs) after transient stimulation with these factors. IL1β would be expected to lead to a change in the expression of inflammatory modifying cytokines. SDF1 was chosen as it is known for its effects on chemotaxis, but little is known about whether the factor changes the phenotype of the cells as well. GCSF and CSF have been proposed in rabbits to be involved in proliferation and self-renewal (Tang *et al.,* 2009), and so would be expected to cause the release of mitogenic factors.

Materials and Methods

Human bone marrow MSCs isolation
Human bone marrow aspirates were obtained from the iliac crest of nine patients following ethical approval (KEK-ZH-Nr: 2010-04444/0) and with the written consent of donors. Nine donors were used (Mean age: 43 years; range: 18-65 years; 7 females and 2 males, Table 1). Mesenchymal stem cells (MSCs) were isolated and expanded as previously described (Li *et al.*, 2009). In brief, the mononuclear cell fraction of whole marrow aspirate was isolated using a Ficol density gradient. Isolated cells were seeded at a density of ~50,000 cells/cm^2 in α-Minimal Essential Medium (αMEM, Gibco/Life Technologies) medium with 10% MSC tested serum (Biochrom/Merck, Darmstadt, Germany) + 5 ng/mL basic fibroblast growth factor (bFGF,

Table 1. Human bone marrow-derived MSCs' donor information.

Donor #	Gender	Age
1	female	65
2	male	41
3	female	64
4	female	40
5	female	18
6	female	20
7	female	40
8	male	41
9	female	55

Fitzgerald Industries International). Cells were left to attach for 4 days and the medium is then replaced three times per week. Cells were harvested by trypsinisation at 60-80 % confluence and reseeded at 3000 cells/cm^2. Cells from all donors were tested to confirm differentiation potential into chondrocytes, adipocytes and osteoblasts (data not shown).

MSCs stimulation
MSCs (6.082 ± 0.83 population doublings after cryopreservation) were plated at a density of 15,000 cells/cm^2 in cell culture polystyrene flasks and cultured in Dulbecco's Modified Eagle's Medium (DMEM) low glucose (Gibco/Life Technologies/Thermo Fisher Scientific, Zug, Switzerland) supplemented with 10 % FCS (Gibco/Life Technologies) and 1 % penicillin/streptomycin (Gibco/Life Technologies) overnight. Two experimental designs for cell stimulation were applied as presented in Fig. 1. On the day of stimulation, medium was aspirated and fresh medium containing 10 ng/mL IL1β, 100 ng/mL SCF, 100 ng/mL GCSF or 1 μg/mL SDF1 (all from PromoCell, Heidelberg, Germany) was added. As negative controls, cells were kept in medium without any factor. After 2 h, medium was aspirated and cells were washed with phosphate buffered saline (PBS) to ensure complete removal of the factors.

An initial screen at the mRNA level was performed at 72 h to ensure an effect of short-term stimulation could be observed. In this initial set of experiments, cells were maintained at 37 °C and 5 % CO$_2$ in DMEM low glucose with 10 % foetal calf serum (FCS) (to prevent cell death) and 1 % penicillin/streptomycin. A second set of experiments to investigate mRNA expression and secreted protein analysis, was also carried out. In the second set of experiments, cells were cultured for 48 h at 37 °C and 5 % CO$_2$ in serum-free DMEM low glucose with 1 % penicillin/
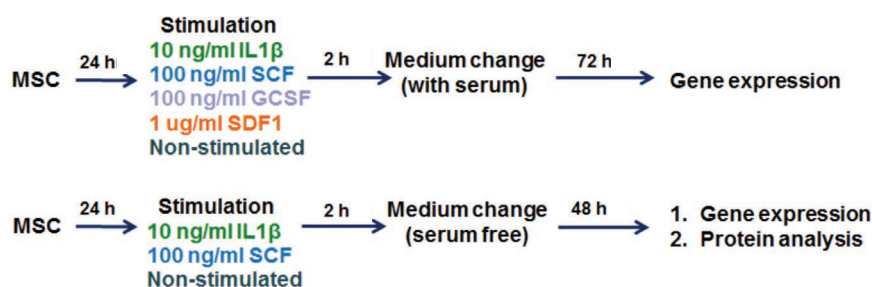

Fig. 1. Experimental outline.

streptomycin to reduce protein contamination from the serum in the secretome analysis (Fig.1).

RNA extraction and reverse transcription

RNA was extracted from cells with TRI reagent followed by on-column method (Qiagen, Hombrechtikon, Switzerland) as previously described (Czekanska *et al.*, 2013). After the extraction, RNA samples were treated with RQ1 (RNA-Qualified) RNase-free DNase to ensure very high quality of RNA samples. Manufacturers' protocol was followed for DNase digestion. After the reaction, the enzyme and buffer components were removed with on-column RNA Clean-Up protocol (Qiagen). Reverse transcription of samples designated for the gene expression analysis with RT² PCRArrays was performed using RT² First Strand Kit according to the supplied instructions. For the gene expression analysis with Real-Time PCR, complementary DNA (cDNA) was transcribed from 0.5 µg total RNA using the TaqMan Reverse Transcription kit.

Cytokine gene expression analysis with RT² PCRArrays

The RT² *Profiler*™ PCR Array system containing primer assays for 84 cytokine genes of interest was applied to analyse the expression of human cytokine genes. Reactions were prepared according to the manufacturer's instructions and run on StepOne Plus cycler (Applied Biosystems/Life Technologies, Foster City, CA, USA). Each plate was incubated for 10 min at 95 °C and followed by 40 cycles of PCR reaction at 95 °C for 15 s and 60 °C for 1 min. To confirm the absence of nonspecific amplification a dissociation curve was generated (60-95 °C for 20 min). After all reactions, the same threshold was set across all PCR array runs. Data was analysed with the SABiosciences PCR Array Analysis Web-based software. The mean of two housekeeping genes, β-2-microglobulin and RPL13A, was used for calculating ΔC_T. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Real-time PCR

Gene expression was analysed with TaqMan probes (Table. 2). RealTime-PCR was performed using StepOne Plus Real-Time PCR System (Applied Biosystems). The reactions were run for 2 min at 56 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Expression of the genes of interest in samples was normalised to RPL13a. Analysis of relative gene expression was performed using $2^{-\Delta\Delta CT}$ method. Expression levels of genes in MSCs stimulated with IL1β or SCF were made relative to the gene expression in unstimulated MSCs.

Conditioned medium (CM) collection and protein analysis

Medium conditioned by stimulated or unstimulated cells was collected and centrifuged at 1000 x *g* at 4 °C for 20 min. Supernatant was transferred into fresh tubes and stored until required at -20 °C. The analysis of secretome was performed using RayBio® Human Cytokine Antibody Array G-series 2000 (RayBiotech, Norcross, GA, USA), which enables detection and semi-quantitative evaluation of 174 cytokine proteins. Preparation of all reagents and procedure was followed according to the supplied instructions. Proteins present in the CM were detected using GenePix 4000B Microarray Scanner (Axon Instruments; Molecular Devices, Sunnyvale, CA, USA). The results were expressed as *n*-fold signal intensity compared to positive control samples. For further analysis, these results were normalised to the number of cells harvested after the collection of CM from cells stimulated or unstimulated cells. To estimate differences in protein synthesis between groups, *n*-fold change was calculated between stimulated groups and control.

Statistical analysis

The data included for statistical analysis are presented as means ± standard error (SE) of independent experiments. All obtained data underwent statistical analysis using SPSS

Table 2. Primers and probes were purchased from Applied Biosystems, except for Runx2, where the primers and probe were purchased from Mycrosynth, CH.

| Gene	Order Code or Sequence
RPL13a (Housekeeping gene)	Hs_01926559_g1
Alkaline Phosphatase	Hs_00758162_m1
Sox9	Hs_00165814_m1
Interleukin-6 (IL-6)	Hs_00985639_m1
Interleukin-8 (IL-8)	Hs_00174103_m1
Interleukin-10 (IL-10)	Hs_00961622_m1
TNFα	Hs_01113624_g1
Wnt3a	Hs_00263977_m1
Wnt4	Hs_00229142_m1
Wnt5a	Hs_00998537_m1
Wnt7b	Hs_00536497_m1
β-catenin	Hs_00170025_m1
Runx2	forward: AGCAAGGTTCAACGATCTGAGAT reverse: TTTGTGAAGACGGGTTATGGTCAA probe: TGAAACTCTTGCCTCGTCCACTCCG
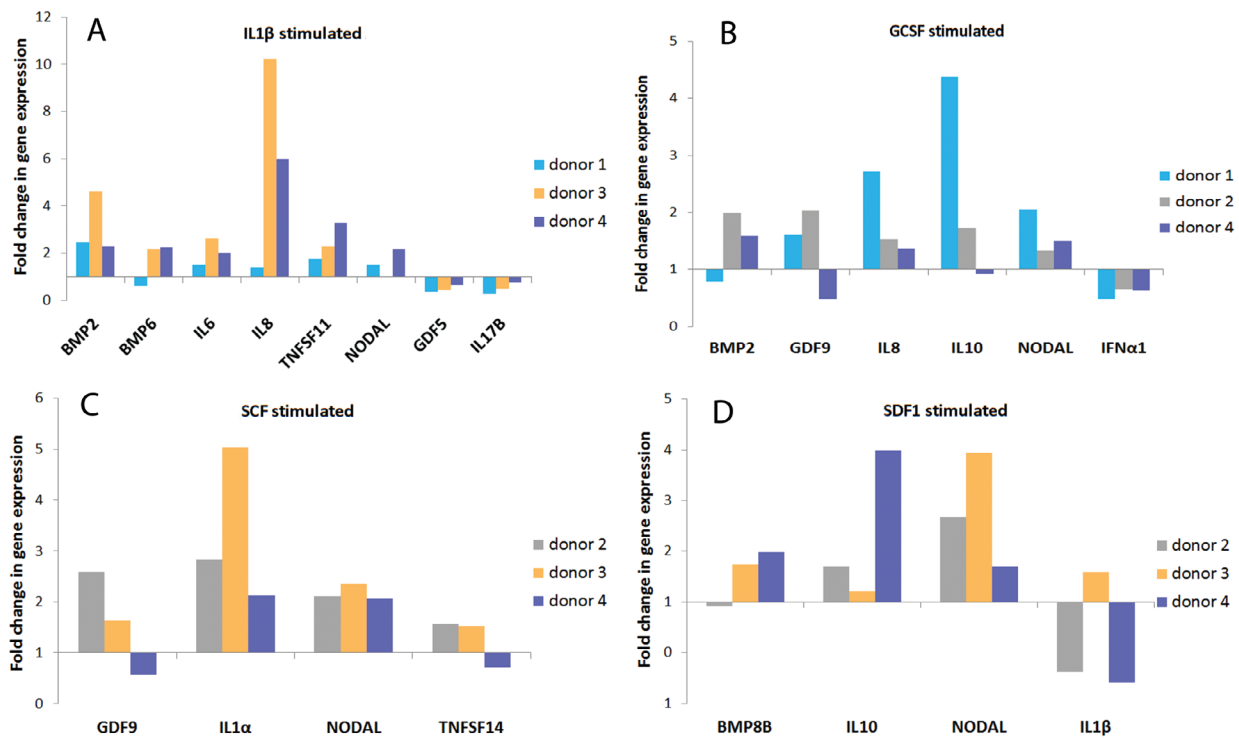
Fig. 2. Diagrams showing the mRNA levels of cytokines in MSC cells stimulated by IL1β (**A**), GCSF (**B**), SCF (**C**) and SDF1 (**D**). Cells in monolayer were stimulated with the specific factor for 2 h. Cells without any stimulation were included as controls for assessing regulatory effects of stimulation. Gene expression level in stimulated cells was expressed relative to control samples.

21.0 (Chicago, IL, USA) software package. Statistical significance at $p < 0.05$ of gene expression in stimulated cells compared to unstimulated cells was determined with Mann-Whitney U test.

Results

Influence of stimulation on cytokine gene expression by MSCs in serum-containing medium

The cytokine and growth factor gene expression analysis in MSC revealed donor dependent response to the stimulation. Yet, consistent effects at the mRNA level 72 h after stimulation were detected. Response to the treatment was assessed as positive if up- or downregulation of a gene occurred in cells obtained from at least 2 out of 3 donors compared to untreated controls ($n = 3$ for each factor and control). Due to differential sensitivity of cells from different donors to the stimulation, results are presented for each individual donor.

A 2 h stimulation of cells with 10 ng/mL IL1β resulted in the upregulation of a wide range of cytokine genes 72 h later. The highest upregulation was detected for BMP2, IL8, TNFSF11 and IL6 in cells from all three donors. BMP6 and NODAL were upregulated in 2 out of 3 donors. In addition, 2 cytokines, GDF5 and IL17B were downregulated in all three donors in response to the treatment (Fig. 2A).

GCSF stimulation resulted in a pronounced upregulation of mRNA expression of 5 cytokines (BMP2, GDF9,

IL10, (2/3 donors) IL8, NODAL (3/3 donors)) and downregulation of one cytokine (INFA1). Cells from all three donors consistently downregulated INFA1 gene expression compared to unstimulated cells (Fig. 2B).

MSC stimulation with 100 ng/mL SCF resulted in high upregulation of IL1A and NODAL in all donors. As seen with GCSF stimulation, cells from donor 4 stimulated with SCF showed the lowest level of response. In addition, two of the donors (1 and 2) positively responded to stimulation by increasing the expression of GDF9 and TNFSF11 compared to unstimulated cells. Interestingly, donor 4, who showed the lowest upregulation in IL1A and NODAL expression out of all donors, showed an opposite response regarding GDF9 and TNFSF11 in comparison to the two other donors (Fig. 2C).

Transient stimulation with SDF1 modified the expression of 4 genes after 72 h. A high upregulation of NODAL gene expression was detected in response to SDF1 treatment in all three donors. Additionally, stimulation had positive effects on two other cytokines, BMP8B and IL10, whereas IL1β was strongly downregulated in 2 out of 3 donors (Fig. 2D). The positive response to SDF1 stimulation in IL1β regulation was detected in cells from donor 3.

Influence of a 2 h IL1β and SCF stimulation on cytokine gene expression by MSC in serum-free medium cell culture

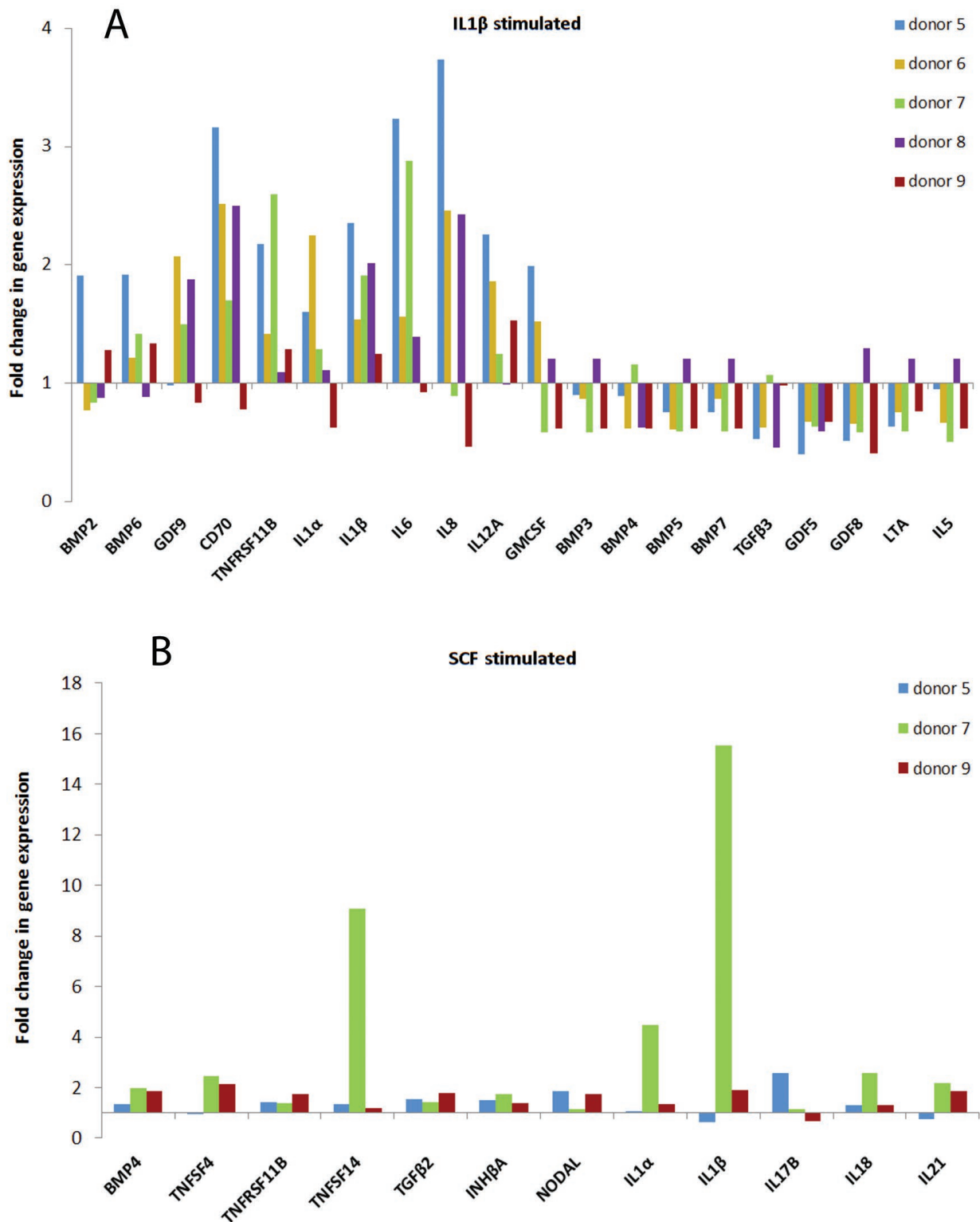In the time between stimulating the cells with either IL1β ($n = 5$) or SCF ($n = 3$) and mRNA sample collection,

Fig. 3. (**A**) mRNA levels of cytokines in MSC cells stimulated with IL1β. Cells in monolayer were stimulated with 10 ng/mL IL1β for 2 h. Cells without any stimulation were included as controls for assessing regulatory effects of stimulation. The gene expression level from stimulated cells was made relative to control samples. (**B**) mRNA levels of cytokines in MSC cells stimulated with SCF. Cells in monolayer were stimulated with 100 ng/mL SCF for 2 h. Cells without any stimulation were included as controls for assessing regulatory effects of stimulation. The gene expression level from stimulated cells was made relative to control samples.
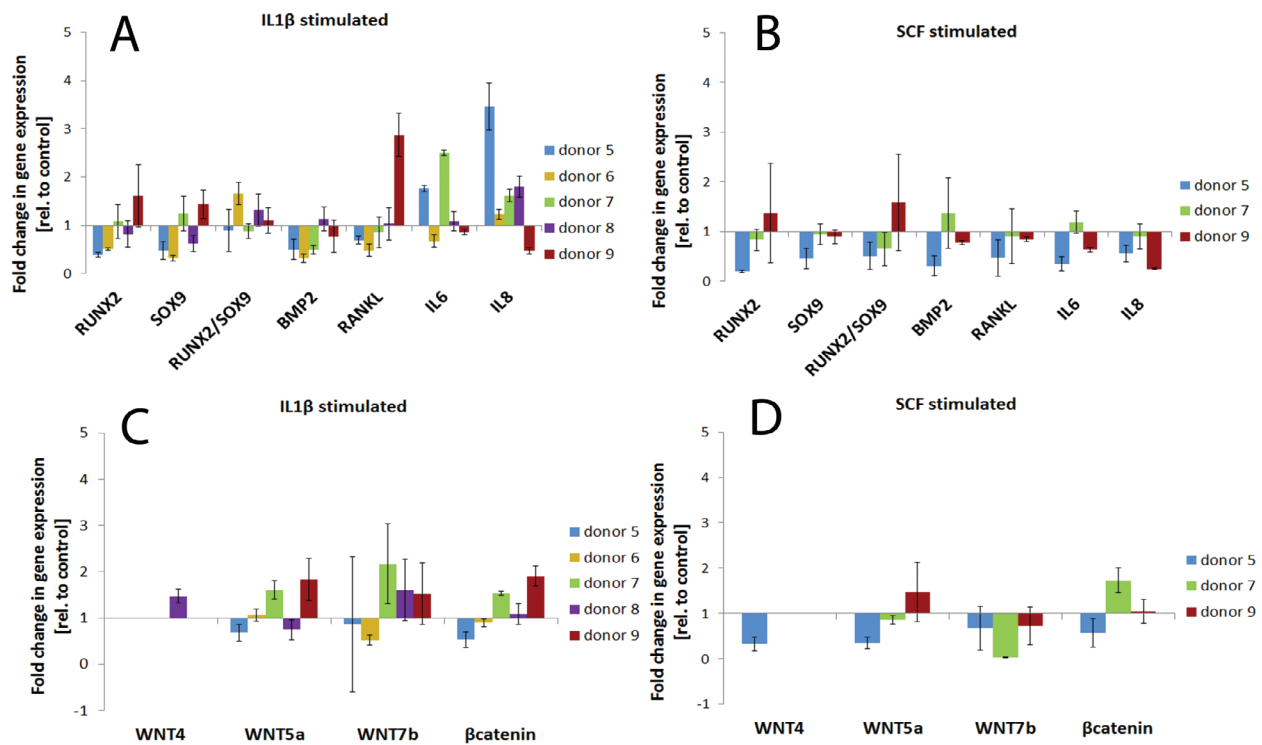
Fig. 4. Diagrams show the fold change gene expression in relative to control from each donor with the standard deviation, quantified by real-time PCR 48 h after the stimulation of MSC with IL1 β or SCF for 2 h. (**A**, **C**) Cells stimulated with IL1β; (**B**, **D**) Cells stimulated with SCF. Cells from 5 and 3 donors were stimulated with IL1β or SCF, respectively.

some of the samples were maintained in serum-free DMEM medium. Forty eight hours after the stimulation with IL1β, mRNA expression of 20 cytokine genes were similarly regulated in at least of 3 out of 5 donors (Fig. 3A). Stimulation of MSCs with IL1β resulted in an upregulation of TNFRSF11B and IL1β and a downregulation of GDF5 gene expression in all 5 donors (Fig. 3A). An increased mRNA expression in stimulated cells was detected for: BMP6, CD70, IL1α, IL6, IL8 and IL12A in 4 out of 5 donors. On the other hand, various BMP-family members (BMP3, 4, 5 and 7), TGFβ3, GDF8, LTA and IL5 were downregulated in 4 donors. A positive response in GDF9 and GMCSF gene regulation was detected in cells from 3 donors. Similarly, 3 out of 5 donors downregulated BMP2 gene expression after 2 h stimulation with IL1β (Fig. 3A).

MSC from 3 donors were treated for 2 h with SCF. As a result, 12 cytokine genes showed response to the stimulation in at least 2 donors (Fig. 3B.). Eight of these genes were upregulated in cells from all 3 donors. More specifically, the mRNA level in stimulated cells relative to unstimulated cells was higher for BMP4, TNFRSF11B, TNFSF14, TGFβ2, INHBA, NODAL, IL1α and IL18. Due to an opposite response in regulation of TNFSF4, IL1β, IL17β and IL21 in cells from donor 5 or 9 (compared to other donors), these genes were upregulated only in 2 out of 3 donors (Fig. 3B).

It has to be highlighted that overall the magnitude of the response was lower compared to the effect of IL1β on cytokine gene regulation. Approximately a 2-fold increase

in gene regulation, relative to unstimulated controls, was detected after SCF stimulation. As an exception, cells from donor 7 were the most sensitive to SCF stimulation, which was marked by the highest increase of TNFSF14, IL1α, IL1β, IL18, IL21 and TNFSF4 mRNA level among all 3 donors (Fig. 3B).

Regulation of the expression of osteogenic and Wnt signalling genes in MSCs stimulated with IL1β and SCF

The gene expression analysis of IL6, IL8, BMP2, RANKL was conducted to confirm the results obtained from RT^2 PCR array analysis (Fig. 4). Additionally, the components of the canonical and non-canonical Wnt signalling pathways, and osteogenic markers (Sox9, Runx2) were assessed at the mRNA level (Fig. 4). Similar to results from the cytokine gene expression arrays, the gene regulation in response to the stimulation was donor dependent. Out of 5 donors, cells from donor 9 showed the opposite response to IL1β treatment than cells from other donors included in the analysis (Fig. 4A,C). Cells from the 4 other donors (donors 5-8) showed a similar trend in the response to IL1β. Hence, donor 9, as an outlier, was excluded from the statistical analysis. For stimulation with SCF, cells from all donors were included for the analysis of any significant differences in response to the stimulation.

IL1β stimulation significantly downregulated mRNA expression of bone markers, including Runx2 ($p = 0.02$), Sox9 ($p = 0.02$), BMP2 ($p = 0.01$) and RANKL ($p = 0.01$)
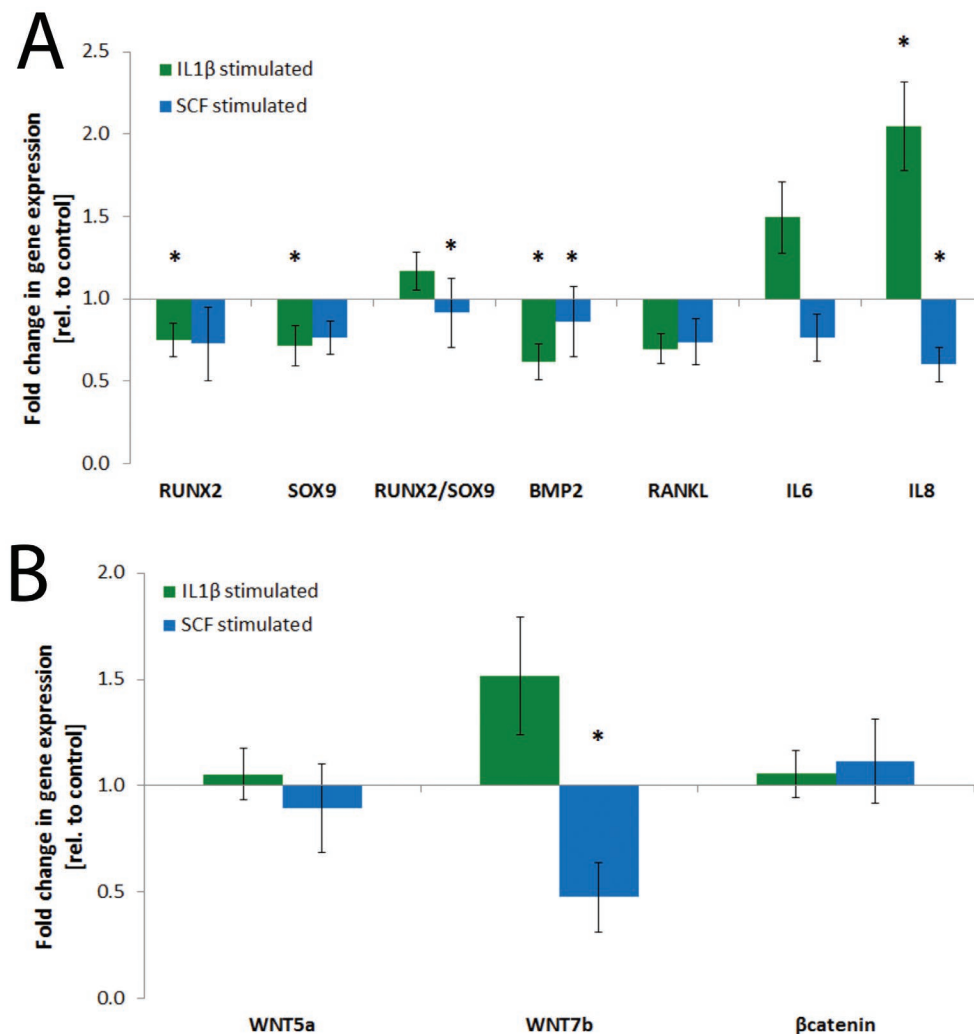
Fig. 5. Diagrams show the mean fold change gene expression relative to control with the standard error of the mean, quantified by real-time PCR 48 h after the stimulation of MSC with IL1β or SCF for 2 h. (**A**) Expression of genes: Runx2, Sox9, Runx2/Sox9, BMP2, RANKL, IL6, IL8; (**B**) Expression of Wnt5a, Wnt7b and β-catenin. Gene expression was normalised to RPL13a and made relative to expression of genes in unstimulated cells. Statistical significance at $p < 0.05$ (*) of genes compared to unstimulated cells was determined with Mann-Whitney U test.

(Fig. 5A). IL1β stimulation had no effect on the overall Runx2/Sox9 ratio compared to unstimulated cells ($p = 0.468$). Treatment with IL1β increased the expression of IL6 and IL8. Yet, this increase was significant only for IL8 ($p < 0.001$). Stimulation with SCF showed a negative effect on the regulation of Runx2, Sox9, BMP2, RANKL, IL6 and IL8 genes (Figs. 4B,5A). This decreased expression reached significance for Sox9 ($p = 0.05$), RANKL ($p = 0.004$) and IL8 ($p = 0.004$) (Fig. 5A).

It was only possible to detect Wnt4 expression in cells from donor 8 and in donor 5 after the stimulation with IL1β and SCF (Fig. 4C,D). Moreover, mRNA expression of Wnt3 was not detected in any donor. Although, there were no significant differences in gene expression of Wnt5a ($p = 0.748$), Wnt7b ($p = 0.3$) or β-catenin ($p = 0.748$) between stimulated with IL1β and unstimulated cells, there was a trend of higher expression of Wnt7b compared to control (Fig. 5B). Stimulation of MSCs with SCF had no

influence on the mRNA expression of Wnt5a ($p = 0.256$) or β-catenin ($p = 0.73$), whereas it significantly decreased Wnt7b expression ($p = 0.008$) compared to control (Fig. 5B).

Influence of stimulation on cytokine protein secretion by MSCs in serum-free medium cell culture

The regulation of 174 cytokine proteins in response to IL1β or SCF stimulation was highly donor dependent, which prevented presentation of averaged results. Nevertheless, it was possible to analyse results in a manner similar to the gene expression results.

The initial analysis of the fluorescence intensity of the medium samples collected from unstimulated cells revealed the secretion of 139 proteins by cells from donor 5, 152 from donor 6 and 9, 154 from donor 7 and 153 from donor 8 (Table 3). For both treatments, the expression of
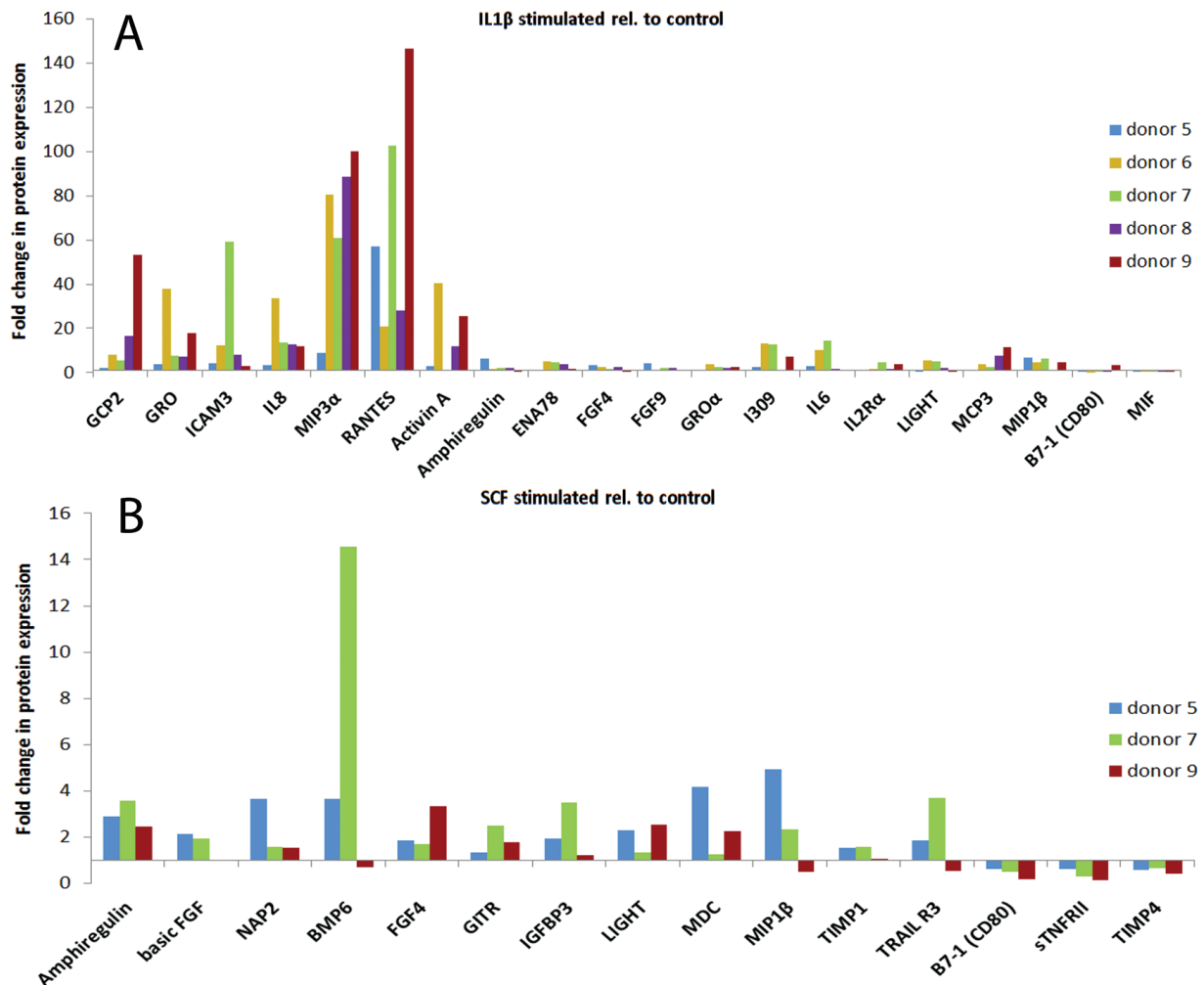
Fig. 6. (**A**) Protein levels of cytokines in medium conditioned by MSCs stimulated with IL1β. Monolayer cells were stimulated for 2 h with 10 ng/mL IL1β. After stimulation, cells were washed with DMEM and maintained in DMEM with 1 % penicillin/streptomycin for 48 h. After that time conditioned medium was collected for the analysis of secreted proteins. (**B**) Protein levels of cytokines in medium conditioned by MSCs stimulated with SCF. Monolayer cells were stimulated for 2 h with 100 ng/mL SCF. After stimulation, cells were washed with DMEM and maintained in DMEM with 1 % penicillin/streptomycin for 48 h. After that time conditioned medium was collected for the protein secretion analysis.

proteins in conditioned medium (CM) was made relative to protein secretion into medium by unstimulated (control) cells. In case of IL1β stimulation, if cells from at least 3 out of 5 donors showed similar regulation of proteins, the effect of stimulation was assessed as positive. Similar conclusions were made for SCF stimulation, when cells from at least 2 out of 3 donors responded to stimulation in similar fashion. All regulated cytokine proteins were grouped depending on their function (Fig. 7), showing that the 2 h stimulation with IL1β mostly influenced the secretion of proteins involved in chemotaxis, inflammation, angiogenesis and bone formation. The stimulation with SCF affected mitogenic, ECM and differentiation markers.

Stimulation with IL1β influenced protein synthesis and secretion in MSC cells. Specifically, 18 out of 174 analysed proteins were upregulated in the conditioned medium collected at 48 h from cells stimulated with IL1β and compared to conditioned medium from unstimulated

cells (Fig. 6A). A lower secretion of 2 proteins, B7-1 and MIF, was observed in response to IL1β stimulation. Out of the upregulated 18 proteins, 9 of them, GCP2, GRO, ICAM3, IL8, MIP3α, RANTES, I309, IL6, IL2Rα and MCP3, were upregulated in cells isolated from all 5 donors. This strongly indicates the regulatory role of IL1β on these cytokines. An increased expression of a further 9 proteins, including Activin A, Amphiregulin, ENA78, FGF4, FGF9, GROα and LIGHT, was detected 48 h after IL1β stimulation in at least 3 of 5 donors (Fig. 6A).

Among 174 proteins analysed from medium conditioned by MSCs stimulated with SCF, secretion of 15 of them were specifically regulated in response to the stimulation (Fig.6B). In relation to results from controls, SCF stimulation showed a negative effect on B7-1, sTNFRII and TIMP4 protein secretion in cells from all 3 donors. On the other hand, all donors showed a positive response to SCF stimulation by increased secretion of Amphiregulin, NAP2,

Function	Cytokine
Chemotactic	GCP2, RANTES, MIP1β, MIP3α, IL2Rα, ENA78, IL8, MDC, B7-1
Mitogenic	Amphiregulin, NAP2, IGFBP3, basic FGF, FGF4, sTNFRII
Proinflammatory	GRO, I309, GROα, ICAM3
Immunoregulation	MIF, IL8, IL2Rα, GITR, LIGHT
Bone formation	FGF4, Amphiregulin, Activin A, IL6, IGFBP3
Angiogenesis	GCP2, FGF9, IL6, IL8, RANTES, ENA78 *(indirectly)*
Chondrogenesis	BMP6
ECM regulation	TIMP1, TIMP4

Fig. 7. Functional grouping of cytokines regulated by 2 h stimulation of bone marrow derived MSC cells with 10 ng/mL IL1β or 100 ng/mL SCF. Colours indicate response to the stimulation: **dark green** – upregulation after stimulation with 10 ng/mL IL1β, light green – downregulation after 10 ng/mL IL1β stimulation, dark blue – upregulation after stimulation with 100 ng/mL SCF, light blue – downregulation, **dark brown** – upregulation by 10 ng/mL IL1β and 100 ng/mL SCF, **light brown** – downregulation by 10 ng/mL IL1β and 100 ng/mL SCF. B7-1 = CD80 – cluster of differentiation 80; basic FGF – basic fibroblast growth factor; BMP4 – bone morphogenic protein 4; BMP6 – bone morphogenic protein 6; ENA78/CXCL5 – epithelial-derived neutrophil-activating peptide 78; FGF4 – fibroblast growth factor 4; GCP2 – granulocyte chemotactic protein; GITR/TNFRSF18 – glucocorticoid induced tumour necrosis factor receptor family related gene; GRO – growth-related oncogene; GROα – growth-related oncogene alpha; I309 = CCL1 – chemokine (C-C motif) ligand 1; ICAM3/CD50 – intercellular adhesion molecule 3; IGFBP3 – insulin-like growth factor binding protein-3; IL2Rα – interleukin 2 receptor alpha; IL1β – interleukin 1 beta; IL6 – interleukin 6; IL8 – interleukin 8; LIGHT = TNFSF14 – tumour necrosis factor ligand superfamily member 14; MIF – Macrophage migration inhibitory factor; MIP1β/CCL4 – Macrophage inflammatory protein-1 beta; MIP3α – Macrophage inflammatory protein-3alpha; NAP2 – neutrophil-activating protein-2; RANTES/CCL5 – regulated on activation, normal T cell expressed and secreted; SCF – stem cell factor; sTNFRII – soluble tumour necrosis factor receptor type II; TIMP1 – tissue inhibitor of metalloproteinase-1; TIMP4 – tissue inhibitor of metalloproteinase-4.

FGF4, GITR, IGFBP3, LIGHT, MDC, TIMP1. Basic FGF, BMP6, MIP1beta and TRAILR3 were upregulated in 2 out of 3 donors, with donor 9 being the exception (Fig. 6B).

Discussion

Immediately after tissue injury or trauma an inflammation response occurs, during which various molecules are released. These factors influence the behaviour and potential of cells involved in the regenerative process. Here, we investigated the longer term effects of a transient 2 h stimulation, with cytokines present during inflammation, on MSC gene expression and secretome. Few studies have been performed using a transient stimulation of MSCs in order to modify their phenotype. The short-term stimulation of human adipose derived stem cells with BMP2, to enhance osteogenic differentiation, showed superior results in a 3D biphasic calcium phosphate scaffold compared to a monolayer culture (Overman *et al.*, 2013a). This occurred after a 15 min stimulation, suggesting it may be possible to reduce the 2 h used during this study. Moreover, the gene expression of trophic factors was differentially regulated in cells cultured in scaffolds compared to monolayer (Overman *et al.*, 2013b).

mRNA analysis after 72 h of serum containing culture

In the initial set of experiments in serum-containing medium, PCR array results showed that even after only 2 h stimulation of cells with each factor, a pronounced cell response was still detectable 72 h after treatment. This would be the critical engraftment time in a clinical setting. Stimulation with IL1β regulated the mRNA expression of the highest number of cytokine genes, emphasising its multifunctional role. Grouping the cytokines based on function can give some indication as to the overall effect that might be obtained. However, due to the complex interactions between the different cytokines, this would need to be further investigated *in vivo* to establish actual clinical benefit. These studies are currently planned. Cytokines, which responded to the IL1β treatment, take part in bone formation (BMP2, BMP6), angiogenesis (IL6, IL8), osteoclastogenesis (TNFSF11), development (NODAL), chondrogenesis (GDF5) and immunomodulation (IL17B). Specifically, only IL1β regulated the expression of BMP6, IL6, TNSFF11 and GDF5. SCF stimulation showed no effect on the gene expression of cytokines implicated in angiogenesis or bone formation. However, it upregulated genes involved in chondrogenesis (GDF9), immunomodulation (IL1A) and

Table 3. **A-B**. Cytokine protein levels secreted by unstimulated MSCs analysed using RayBio Cytokine Array G-Series. Cells in monolayer were cultured for 48 h in serum-free medium. Protein level secreted by unstimulated MSCs (control) determined by the fluorescence signal intensity: *n.d.* (= not detected) – for the signal intensity up to 10; *low* – signal intensity of 10-1,000; *medium* – signal intensity of 1,001-10,000; *high* – signal intensity above 10,000.

A

Protein	donor 5	donor 6	doonor 7	donor 8	donor 9
Angiogenin	medium	high	high	high	high
BDNF	low	low	low	low	low
BLC	low	low	low	low	n.d.
BMP-4	low	n.d.	n.d.	n.d.	n.d.
BMP-6	n.d.	n.d.	n.d.	n.d.	low
CK beta 8-1	low	low	low	low	n.d.
CNTF	low	low	low	low	low
EGF	n.d.	n.d.	n.d.	n.d.	low
Eotaxin	low	n.d.	n.d.	low	n.d.
Eotaxin-2	n.d.	n.d.	n.d.	n.d.	n.d.
Eotaxin-3	low	low	low	low	low
FGF-6	low	low	low	low	low
FGF-7	low	low	low	low	low
Flt-3 Ligand	low	low	low	low	low
Fractalkine	low	n.d.	n.d.	n.d.	n.d.
GCP-2	low	low	n.d.	low	n.d.
GDNF	low	low	low	low	n.d.
GM-CSF	low	low	low	low	low
I-309	low	n.d.	n.d.	low	n.d.
IFN-gamma	low	low	low	low	low
IGFBP-1	low	low	low	low	n.d.
IGFBP-2	low	low	low	low	low
IGFBP-4	low	low	low	low	n.d.
IGF-I	n.d.	n.d.	n.d.	n.d.	n.d.
IL-10	low	low	low	low	low
IL-13	low	low	low	low	low
IL-15	low	low	low	low	low
IL-16	n.d.	n.d.	n.d.	n.d.	n.d.
IL-1alpha	low	low	low	low	low
IL-1beta	low	low	low	low	low
IL-1ra	low	low	low	low	low
IL-2	low	low	low	low	low
IL-3	low	low	low	low	low
IL-4	n.d.	n.d.	n.d.	n.d.	n.d.
IL-5	low	low	low	low	low
IL-6	high	medium	medium	high	high
IL-7	low	low	low	low	low
Leptin	low	low	low	low	low
LIGHT	low	n.d.	n.d.	low	n.d.
MCP-1	high	high	high	high	high
MCP-2	low	low	low	low	low
MCP-3	low	low	low	low	low
MCP-4	low	low	low	low	low
M-CSF	low	low	low	low	low
MDC	low	n.d.	n.d.	n.d.	n.d.
MIG	low	low	low	low	low
MIP-1-delta	n.d.	n.d.	n.d.	n.d.	n.d.
MIP-3-alpha	low	n.d.	n.d.	n.d.	n.d.
NAP-2	low	low	low	low	n.d.
NT-3	low	low	low	low	n.d.
PARC	low	low	low	low	low
PDGF-BB	low	low	low	low	low
RANTES	low	low	low	low	low
SCF	low	low	low	low	low
SDF-1	low	low	low	low	low
TARC	low	low	low	low	n.d.
TGF-beta 1	low	low	low	low	low
TGF-beta 3	n.d.	n.d.	n.d.	n.d.	n.d.
TNF-alpha	low	low	low	low	low
TNF-beta	low	low	low	low	low

B

Protein	donor 5	donor 6	doonor 7	donor 8	donor 9
Acrp30	low	low	low	low	low
AgRP	low	low	low	low	low
Angiopoietin-2	medium	low	low	low	low
Amphiregulin	n.d.	low	low	low	low
Axl	n.d.	low	low	low	low
bFGF	n.d.	low	low	low	low
b-NGF	n.d.	low	low	low	low
BTC	low	low	low	low	low
CCL-28	low	low	low	low	low
CTACK	low	low	low	low	low
Dtk	n.d.	low	low	low	low
EGF-R	low	low	low	low	low
ENA-78	n.d.	low	low	low	low
Fas/TNFRSF6	low	low	low	low	low
FGF-4	n.d.	low	low	low	low
FGF-9	n.d.	low	low	low	low
GCSF	low	low	low	low	low
GITR-Ligand	low	low	low	low	low
GITR	n.d.	low	low	low	low
GRO	medium	medium	medium	medium	medium
GRO-alpha	low	low	low	low	low
HCC-4	low	low	low	low	low
HGF	medium	medium	n.d.	medium	high
ICAM-1	n.d.	low	low	low	low
ICAM-3	n.d.	low	n.d.	low	low
IGFBP-3	n.d.	low	low	low	low
IGFBP-6	low	low	medium	medium	medium
IGF-I SR	medium	low	low	low	low
IL-1 R4/ST2	low	low	low	low	low
IL-1 RI	low	low	low	low	low
IL-11	low	low	low	low	low
IL-12 p40	n.d.	low	low	low	low
IL-12 p70	n.d.	low	low	low	low
IL-17	low	low	low	low	low
IL-2 Rapha	low	low	low	low	low
IL-6 R	n.d.	low	low	low	low
IL-8	low	low	low	low	low
I-TAC	low	n.d.	low	low	low
Lymphotactin	n.d.	low	low	low	low
MIF	medium	low	medium	low	low
MIP-1alpha	n.d.	n.d.	low	low	low
MIP-1beta	n.d.	low	low	low	low
MIP-3beta	n.d.	low	low	low	low
MSP-alpha	low	n.d.	low	low	low
NT-4	low	low	low	low	low
Osteoprotegerin	high	high	high	high	high
Oncostatin M	low	low	low	low	low
PlGF	low	low	low	low	low
sgp130	low	low	low	low	low
sTNF RII	n.d.	low	low	n.d.	low
sTNF-RI	low	low	medium	medium	medium
TECK	n.d.	low	low	low	low
TIMP-1	medium	medium	medium	medium	medium
TIMP-2	high	high	high	high	high
Thrombopoietin	low	low	low	low	low
TRAIL R3	n.d.	low	low	low	low
TRAIL R4	low	low	low	low	low
uPAR	low	medium	low	low	low
VEGF	low	low	low	low	low
VEGF-D	n.d.	low	low	low	low

Table 3. **C**. Cytokine protein levels secreted by unstimulated MSCs analysed using RayBio Cytokine Array G-Series. Cells in monolayer were cultured for 48 h in serum-free medium. Protein level secreted by unstimulated MSCs (control) determined by the fluorescence signal intensity: *n.d.* (= not detected) – for the signal intensity up to 10; *low* – signal intensity of 10-1,000; *medium* – signal intensity of 1,001-10,000; *high* – signal intensity above 10,000.

Protein	donor 5	donor 6	doonor 7	donor 8	donor 9
Activin A	low	low	low	low	low
ALCAM	low	low	low	n.d.	low
B7-1(CD80)	n.d.	low	low	n.d.	low
BMP-5	low	low	low	low	low
BMP-7	low	low	low	low	low
Cardiotrophin-1	low	low	low	low	low
CD14	low	n.d.	low	n.d.	low
CXCL- 16	low	low	low	low	low
DR6 (TNFRSF21)	low	low	low	low	low
Endoglin	low	low	low	low	low
ErbB3	low	low	low	low	low
E-Selectin	low	low	low	low	low
Fas Ligand	low	low	low	low	low
ICAM-2	low	low	low	low	low
IGF-II	medium	medium	medium	medium	medium
IL-1 R II	low	low	low	low	low
IL-10 Rbeta	low	low	low	low	low
IL-13 Ralpha2	low	low	low	low	low
IL-18 BPalpha	low	low	low	low	low
IL-18 Rbeta	low	low	low	low	low
IL-2 Ralpha	low	low	low	low	low
IL-2 Rbeta	low	low	low	low	low
IL-2 Rgamma	low	low	low	low	low
IL-21R	low	low	low	low	low
IL-5 Ralpha	low	low	low	low	low
IL-9	low	low	low	low	low
IP-10	low	low	low	low	low
LAP	medium	medium	medium	medium	medium
Leptin R	low	low	low	low	low
LIF	low	low	low	low	low
L-Selectin	low	low	low	low	low
M-CSF R	low	low	low	low	low
MMP-1	low	low	low	low	low
MMP-13	low	low	low	n.d.	low
MMP-9	n.d.	low	low	n.d.	low
MPIF-1	low	low	low	low	low
NGF R	low	low	low	low	low
PDGF AA	low	low	low	low	low
PDGF-AB	low	low	low	low	low
PDGF Ralpha	low	low	low	low	low
PDGF Rbeta	low	low	doonor	low	low
PECAM-1	n.d.	n.d.	n.d.	n.d.	low
Prolactin	low	low	low	low	low
SCF R	low	low	low	low	low
SDF-1beta	low	low	low	low	low
Siglec-5	low	low	low	low	low
TGF-alpha	n.d.	n.d.	n.d.	n.d.	low
TGF beta2	low	low	low	low	low
Tie-1	low	low	low	low	low
Tie-2	low	low	low	low	low
TIMP-4	low	n.d.	low	n.d.	low
VE-Cadherin	low	low	low	low	low
VEGF R2	low	low	low	low	low
VEGF R3	low	low	low	low	low

development (NODAL). Short-term stimulation of MSCs with GCSF positively regulated the response at the mRNA level of cytokines implicated in bone formation (BMP2), chondrogenesis (GDF9), angiogenesis (IL8, IL10) and development (NODAL) while it decreased the expression of IFNA1, which is involved in immunological responses. SDF1 stimulation uniquely regulated gene expression of the bone formation factor, BMP8b. Additionally, it stimulated the expression of an angiogenic factor (IL10) and downregulated expression of IL1β. Similarly, as with stimulation with other factors, a cytokine involved in developmental processes, NODAL, was upregulated in response to SDF1 stimulation. The regulation of different genes indicates that the potential of MSCs can be modulated depending on the aim of the application. Interestingly, the synergic effects of SCF and GCSF on stimulating proliferation and inhibiting differentiation of rabbit MSCs have been reported (Tang *et al.*, 2009). It would be of interest to analyse whether the combined stimulation of MSCs with all or some of factors included in this study synergises and results in an even stronger response.

mRNA and secretome analysis after 48 h of serum-free culture

Due to the high interest in IL1β-regulated osteogenic differentiation (Lee *et al.*, 2010; Mumme *et al.*, 2012; Sonomoto *et al.*, 2012; Ferreira *et al.*, 2013; Loebel *et al.*, 2014), and recent promising influence of SCF on tissue regeneration (Kim *et al.*, 2011; Pan *et al.*, 2013), our further investigations focused on a more detailed analysis of the direct effects of these two cytokines. We were particularly interested in regulation of cytokine genes and protein expression that could provide more predictable indications regarding the effects triggered by IL1β or SCF. To ensure that changes detected were specific for the stimulating factor used, and to allow for a more accurate protein analysis without contaminating serum proteins, the further investigations were performed after MSC stimulation in a serum-free medium for 48 h. Longer periods of serum-free culture were not possible due to signs of cell distress.

Interestingly, more pronounced effects on cytokine gene expression were detected at 48 h serum-free culture compared to results of the analysis made 72 h serum containing culture. After IL1β stimulation, 11 cytokine genes were upregulated and 9 cytokine genes were downregulated (Fig. 3A). BMP2, BMP6, IL6, IL8 and GDF5 showed the same response to IL1β treatment 48 and 72 h after the stimulation.

SCF resulted in the upregulation of 13 cytokine genes (Fig. 3B). Only TNFSF14 and IL1α, but not GDF9 and NODAL gene expression were upregulated 48 h after SCF stimulation, whereas all of these cytokines were upregulated 72 h after SCF treatment. These partial variances in the response to stimulation could be attributed to the differences in the time of the analysis of gene expression, *i.e.*, 48 or 72 h, after the stimulation. It is highly probable that the expression of some of the genes increases or diminishes within a 24 h frame between both time points. In addition, the presence of serum in the initial set of experiments may also modify any response seen.

The consistency of the data obtained from both serum containing and serum-free culture, increases the confidence in the changes observed. However, where differences were seen it must be taken into consideration that data from donors 1-4 was obtained during the serum containing 72 h experiment, while donors 5-9 were used for the serum-free 48 h experiments.

IL1β and SCF regulated gene expression of interleukin family members (Fig. 3). These cytokines are recognised for their pro-inflammatory role and destructive effects on bone in pathological conditions including osteoarthritis (Fernandes *et al.*, 2002; Kapoor *et al.*, 2011). Paradoxically, in the context of bone regeneration, the presence of some of these pro-inflammatory cytokines promotes the bone regeneration in multiple ways. Importantly, this positive effect strongly depends on the timing of their expression. Both, IL12 and IL18 inhibit *in vitro* osteoclast differentiation. In addition, IL18 directly induces production of IL6, which is known for its potent roles at early stages of the bone healing process (Gracie *et al.*, 2003). In fact, IL6 together with IL8 are the major angiogenic factors stimulating vascular endothelial growth factor (VEGF) during fracture healing (Martin *et al.*, 2009; Kolar *et al.*, 2011). IL1β treatment increased the gene and protein expression of IL6 and IL8, whereas SCF had an opposite effect on gene expression of these cytokines, suggesting the involvement of IL1β, but not SCF, in triggering angiogenesis. On the other hand, the stimulatory effects of SCF on angiogenesis could potentially be mediated through the upregulation of IL18 cytokine gene expression inducing IL6 (Gracie *et al.*, 2003).

The gene expression of BMP superfamily members was lower in cells after IL1β treatment (Fig. 3A). BMPs are important for proliferation, osteoblastic and chondrogenic differentiation, morphogenesis and apoptosis. A heterodimeric association of BMP4/7 and BMP2/7 regulates the proliferation of MSCs and induces the differentiation of MSCs into osteoblastic lineage (Tsiridis *et al.*, 2007). Similarly, BMP5 and TGFβ$_3$ possess osteoinductive properties (Wozney, 2002). At the same time, BMP6, a factor implicated in chondrocyte maturation, was upregulated after the stimulation with IL1β. GDF5 and GDF8 are involved in chondrogenesis and callus formation. Additionally, GDF8, a negative regulator of skeletal muscle growth, stimulates the adipogenic differentiation in MSCs, whereas its absence increases osteogenic differentiation (Artaza *et al.*, 2005; Hamrick *et al.*, 2007). Interestingly, a positive correlation of GDF8 deficiency and fracture callus size in a mouse *in vivo* model was reported (Kellum *et al.*, 2009).

In addition to the assessment of cytokine genes in response to 2 h stimulation of cells with IL1β and SCF, the expression of Wnt signalling pathway components and their relation to Sox9 and Runx2 transcription factor gene expression was analysed after 48 h of serum-free culture post treatment. The analysis of Wnt 3a, 4, 5a, 7b and β-catenin mRNA expression in cells stimulated with IL1β or SCF relative to untreated cells was strongly donor dependent. This donor variation was also seen in the expression changes of other genes. The investigation of a stimulus on multiple donors is required in order to

determine the frequency with which a response is observed. This is turn provides an indication as to its potential future clinical applicability.

In the experimental setting within this study, the expression of Wnt3a was not detected in cells, neither with nor without IL1β or SCF stimulation. Wnt4 gene expression was detectable in control samples and in treated cells from 2 donors (5 and 8); whereas Wnt5a, 7b and β-catenin were expressed in stimulated and unstimulated cells. Both Wnt3a and Wnt4, together with β-catenin, are involved in the canonical Wnt pathway stimulating the expression of alkaline phosphatase (ALP), osteocalcin (OC), BMP2 and Runx2 (Kim *et al.*, 2013), and inhibiting the non-canonical Wnt pathway (Boland *et al.*, 2004). Importantly, the effect of canonical Wnt signalling on osteogenesis is highly dependent on the stage of target cells (Ling *et al.*, 2009). Wnt5a and Wnt7b activate the non-canonical Wnt signalling pathway inducing several cascades, including planar-cell polarity (PCP), c-Jun N-terminal kinase (JNK), Nemo-like kinase (NLK) and Rho-associated kinase (ROK) signalling (Ling *et al.*, 2009). Our results indicated that although the stimulation with IL1β had no significant effect on Wnt5a, Wnt7b and β-catenin gene expression, Wnt7b showed a trend of increased expression upon the treatment with IL1β. This was in conjunction with a significant decrease in Runx2 and Sox9 gene expression. The treatment with SCF showed similar trends with significantly downregulated Wnt7b. The canonical Wnt signalling stimulates the differentiation of MSCs committed to osteogenic lineage, whereas it suppresses the terminal differentiation of mature osteoblasts (Ling *et al.*, 2009). The former process is promoted through the upregulation of Runx2, which along with Sox9, is a transcriptional effector of the canonical Wnt signalling (Parr *et al.*, 1993; Eames *et al.*, 2004). Recent evidence showed that the promotion of osteogenic differentiation by IL1β is dependent on the non-canonical Wnt pathway stimulating Runx2 gene expression (Arnsdorf *et al.*, 2009; Sonomoto *et al.*, 2012). Although the expression of Runx2 and Sox9 was downregulated upon the stimulation of MSCs with IL1β or SCF, the Runx2/Sox9 ratio remained at the same level suggesting that the short term treatment induces other mechanisms and outcomes in MSCs osteogenic differentiation in a monolayer culture.

Secretome analysis revealed increased levels of proteins involved in the bone formation process (Fig. 7), in response to the short-term stimulation with IL1β or SCF.

We observed an interesting association between the results from PCR and protein arrays regarding the expression and regulation of factors involved in fracture healing and bone remodelling. The results from post stimulation serum-free cultures showed the mRNA expression of Receptor activator of NF-κβ ligand (RANKL) was downregulated, whereas at the same time TNFRSF11b was upregulated in response to the stimulation with IL1β or SCF. RANKL is a paracrine factor involved in osteoblast-osteoclast progenitor cells cross-talk. Secreted RANKL protein binds to RANK receptor on the osteoclast progenitor cell surface activating the differentiation of the osteoclast phenotype (Udagawa *et al.*, 1999). Interestingly, TNFRSF11b is a gene coding the osteoprotegerin (OPG)

protein, an inhibitor of osteoclastogenesis and activation of mature osteoclasts. In detail, OPG is the decoy receptor for RANKL expressed and secreted by osteoblasts and osteoprogenitor cells. The regulation of both OPG and RANKL is Wnt signalling dependent (Maeda *et al.*, 2012; Maeda *et al.*, 2013). Although, the protein level of OPG in the conditioned medium 48 h after the stimulation of MSCs with IL1β or SCF was at the same level as in untreated cells (data not shown), based on the mRNA expression level results, short term stimulation with IL1β or SCF strongly suggest the indirect inhibitory effects of these factors on the osteoclasts differentiation. Additionally, we reported Wnt pathway dependent regulation of OPG and RANKL in response to IL1β and SCF. The gene expression of additional factors inhibiting osteoclastogenesis was positively regulated in response to the stimulation. SCF treatment upregulated the expression of IL18, stimulating the production of osteoclast differentiation inhibitor, granulocyte macrophage colony-stimulating factor (GM-CSF), by T-cells (Horwood *et al.*, 1998; Gracie *et al.*, 2003). In the case of short-term stimulation of MSCs with IL1β, the expression of GM-SCF gene was directly upregulated after the treatment.

Understanding the mechanisms involved in the indirect effects of preconditioning, *i.e.*, due to the enhancement of the secretion of trophic factors acting in a paracrine manner, would be of great interest for cell-based regenerative therapies. Especially the relevance of the secreted factors after the stimulation in the context of bone regeneration would aid bone tissue regeneration strategies. Hence, it is crucial to further analyse the indirect effects of short-term stimulation in the context of bone healing within a reliable *in vitro* cell culture model, which in reality should mimic endochondral ossification. In fact, it is challenging to recapitulate endochondral ossification, which occurs during bone formation or fracture healing, in *in vitro* conditions. The conventional monolayer *in vitro* models focus on the direct stimulation of osteogenesis which is alike intramembranous ossification but rarely occurs in the initial phase of fracture healing. The controlled stimulation of chondrogenic differentiation, followed by the osteogenic differentiation of MSCs in a 3D *in vitro* culture, results in formation of bone tissue *via* endochondral ossification (Muraglia *et al.*, 2003; Scotti *et al.*, 2010). Such models may be more suitable for future *in vitro* investigations into fracture healing.

Conclusions

The regeneration of bone is triggered by factors produced during the inflammation phase that occurs after trauma. These factors influence the behaviour of cells residing at the injury site by acting in a direct and indirect manner. Our results indicate the potential of a transient stimulation of cells by a number of factors to enhance the regulation of trophic factors over longer period of time. IL1β strongly promoted the secretion of a wide range of proteins with chemotactic, proinflammatory and angiogenic properties, suggesting it may have a greater influence in the early bone repair environment. Whereas SCF regulated the expression

of proteins involved in proliferation, chondrogenesis and ECM regulation, indicating its use may be more beneficial in either cartilage repair or callus formation. This effect was achieved by a short stimulation, which could be applied as stem cell therapy strategy and performed intra-operatively.

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Editor's Note: All questions/comments by the reviewers were answered by text changes. There is hence no Discussion with Reviewers section.