Supplementary Material

1 Supplementary materials and methods

We investigated the influence of FBS and hPL on osteogenic differentiation and bone-like matrix production in a three-dimensional (3D) MSC mono-culture. The materials and methods used for this part of the study are described below. The results on this part of the study can be found in Figure S4.

1.1 Scaffold fabrication

Bombyx mori L. silkworm cocoons were degummed by boiling them in 0.2 M Na$_2$CO$_3$ for 1 h. After drying, silk was dissolved in 9 M LiBr, filtered, and dialyzed against ultra-pure water (UPW) for 36 h using SnakeSkin Dialysis Tubing (11532541, Thermo Fisher Scientific, Breda, The Netherlands). The dialyzed silk fibroin solution was frozen at -80°C and lyophilized for 7 days. Lyophilized silk fibroin was dissolved in hexafluoro-2-propanol at a concentration of 17% (w/v) and casted in scaffold molds containing NaCl granules with a size of 250-300 µm as template for the pores. Molds were covered to improve the silk fibroin blending with the granules. After 3 h, covers were removed from molds, and hexafluoro-2-propanol was allowed to evaporate for 7 days whereafter β-sheets were induced by submerging silk fibroin-salt blocks in 90% MeOH for 30 min. NaCl was dissolved from the scaffolds in ultra-pure water, resulting in porous sponges. These sponges were cut into scaffolds of 3 mm in height and 5 mm in diameter. Scaffolds were sterilized by autoclavation in PBS at 121°C for 20 min.

1.2 Cell culture

Mesenchymal stromal cells (MSCs) were isolated from human bone marrow (Lonza, Walkersville, MD, USA) and characterized for surface markers and multilineage differentiation, as previously described (1). MSCs were frozen at passage 3 with 1.25*10$^6$ cells/ml in freezing medium containing fetal bovine serum (FBS, BCBV7611, Sigma-Aldrich, Zwijndrecht, The Netherlands) with 10% DMSO and stored in liquid nitrogen until further use. Before experiments, MSCs were thawed, collected in high glucose DMEM (hg-DMEM, 41966, Thermo Fisher Scientific), seeded at a density of 2.5*10$^3$ cells/cm$^2$ and expanded in expansion medium containing hg-DMEM, 10% FBS (BCBV7611, Sigma-Aldrich), 1% Antibiotic Antimyotic (anti-anti, 15240, Thermo Fisher Scientific), 1% Non-Essential Amino Acids (11140, Thermo Fisher Scientific), and 1 ng/mL basic fibroblastic growth factor (bFGF, 100-18B, PeproTech, London, UK) at 37 °C and 5% CO$_2$. After 9 days, cells were detached using 0.25% trypsin-EDTA (25200, Thermo Fisher Scientific) and seeded onto scaffolds at passage 4. Cells were seeded at a density of 10$^6$ cells per scaffold ($N = 4$ scaffolds per condition) and seeding was performed dynamically for 6 hours in 50 ml tubes on an orbital shaker at 150 RPM in expansion medium (2). The cell-loaded scaffolds were cultured for 4 weeks at 37 °C and 5% CO$_2$ in custom-made spinner flask bioreactors and a rotational speed of 300 RPM. MSCs were stimulated to undergo osteogenic differentiation with osteogenic medium containing Ig-DMEM (22320, Thermo Fisher Scientific), 10% FBS (SFBS, Bovogen, East Keilor, Australia) or 10%, 5%, or 2.5% human platelet lyste (hPL, PE20612, PL BioScience, Aachen, Germany), 1% anti-anti, 10 mM β-glycerophosphate (G9422, Sigma-Aldrich), 50 µg/ml ascorbic acid-2-phosphate (A8960, Sigma Aldrich), and 100 nM Dexamethasone (D4902, Sigma-Aldrich)). Medium was refreshed 3 times per week and samples were collected and stored at -80 °C on day 7.
1.3 Micro-computed tomography (µCT)

Bioreactors were scanned and analyzed with a µCT100 imaging system (Scanco Medical, Brüttisellen, Switzerland) after 4 weeks of culture. Scanning was performed at an isotropic nominal resolution of 17.2 µm, energy level of 45 kVp, intensity of 200 µA, integration time of 300 ms and with twofold frame averaging. To reduce part of the noise, a constrained Gaussian filter was applied with filter support 1 and filter width sigma 0.8 voxel. Filtered images were segmented to detect mineralization at a global threshold of 24% of the maximum grayscale value. Unconnected objects smaller than 30 voxels were removed through component labeling.

1.4 (Immuno)histochemistry

Scaffolds (N = 2) were soaked for 15 minutes in each 5% (w/v) sucrose and 35% (w/v) sucrose in phosphate buffered saline (PBS). Samples were embedded in Tissue Tek® (Sakura, Alphen aan den Rijn, The Netherlands) and quickly frozen with liquid N2. Cryosections were sliced with a thickness of 5 µm. Upon staining, sections were fixed for 10 minutes in 3.7% neutral buffered formaldehyde and washed twice with PBS.

To visualize collagen deposition, sections were stained with Picrosirius Red. Sections were soaked in Weigert’s Iron Hematoxylin (HT1079, Sigma-Aldrich) solution for 10 minutes, washed in running tap water for 10 minutes, and stained in 1% w/v Sirius Red (36,554-8, Sigma-Aldrich) in picric acid solution (36011, Sigma-Aldrich) for one hour. Subsequently, sections were washed in two changes of 0.5% acetic acid and dehydrated in one change of 70% and 96% EtOH, three changes of 100% EtOH, and two changes of xylene. Sections were mounted with Entellan (107961 Sigma-Aldrich) and imaged with a bright field microscope (Zeiss Axio Observer Z1, 20x/0.8 Plan-Apochromat objective).

To study osteogenic differentiation, sections were stained with DAPI, CNA35, osteopontin and runt-related transcription factor 2 (RUNX2). Briefly, sections were permeabilized in 0.5% triton X-100 in PBS for 5 min and blocked in 10% normal goat serum in PBS for 30 min. Primary antibodies were incubated overnight at 4 ºC, secondary antibodies were incubated with 0.1 µg/ml DAPI and 1 µmol/mL CNA35-mCherry (3) for 1 h at room temperature. Antibodies are listed in Table S1. Images were acquired with a laser scanning microscope (Leica TCS SP5X, 63x/1.2 HCX PL Apo CS objective). All images were prepared for presentation in Fiji (4).

1.5 DNA quantification

Lyophilized samples (N = 3) were weighted and digested overnight in papain digestion buffer (containing 100 mmol phosphate buffer, 5 mmol L-cystein, 5 mmol EDTA and 140 µg/ml papain (P4762, Sigma-Aldrich)). DNA was quantified using the Qubit Quantification Platform (Q32851, Thermo Fisher Scientific), according to the manufacturer’s instructions.

1.6 Alkaline phosphatase activity

Scaffolds (N = 3) were washed in PBS and disintegrated using steel balls and a mini-beadbeater™ (Biospec, Bartlesville, OK, USA) in cell lysis buffer containing 0.2% (v/v) Triton X-100 and 5 mM MgCl2. Alkaline phosphatase (ALP) activity in cell lysates was determined by adding 20 µl of 0.75 M 2-amino-2-methyl-1-propanol (A65182, Sigma-Aldrich) to 80 µl sample in 96-wells assay plates.
Subsequently, 100 µl substrate solution (10 mM p-nitrophenyl-phosphate (71768, Sigma-Aldrich) in 0.75 M 2-amino-2-methyl-1-propanol) was added and wells were incubated at room temperature for 15 minutes. To stop the reaction, 100 µl 0.2 M NaOH was added. Absorbance was measured with a plate reader at 450 nm and these values were converted to ALP activity (converted p-nitrophenyl phosphate in µmol/ml/min) using standard curve absorbance values.

1.7 Receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) quantification

Secreted RANKL and OPG were quantified in cell supernatants from day 7 of 2 different bioreactors containing 4 scaffolds each (N = 2) with RANKL (ab213841, Abcam, Cambridge, UK) and OPG (EHTNFRSF11B, Thermo Fisher Scientific) enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer’s protocols. To measure RANKL, samples were added to anti-human RANKL coated microwells. After 90 min incubation at 37 ºC, samples were replaced by biotinylated antibody solution followed by 60 min incubation at 37 ºC. After thorough washing, avidin-biotin-peroxidase complex (ABC) solution was added and plates were incubated for 30 min at 37 ºC. Wells were again washed and color developing agent was added followed by 15 min incubation in the dark at 37 ºC. To stop the reaction, stop solution was added and absorbance was measured at 450 nm in a plate reader. To measure OPG, samples were added to anti-human OPG coated microwells and incubated for 2.5 h at room temperature with gentle shaking. Wells were subsequently washed, biotinylated antibody solution was added followed by 60 min incubation at room temperature with gentle shaking. After washing, streptavidin-HRP solution was added and incubated in the wells for 45 min with gentle shaking. Wells were subsequently washed and incubated with substrate solution for 30 min in the dark with gentle shaking. The enzymatic reaction was stopped with stop solution and absorbance was measured at 450 nm in a plate reader. All absorbance values were converted to RANKL and OPG concentrations using standard curve absorbance values.
2 Supplementary Tables

The antibodies that were used for immunofluorescent stainings of MC-MSC co-cultures (non-stimulated and osteogenically stimulated) and three-dimensional osteogenically stimulated MSC mono-cultures are listed in Table S1.

Table S1. List of antibodies that were used in this study.

| Antigen            | Supplier   | Catalogue No. | Conjugate | Species | Dilution |
|--------------------|------------|---------------|-----------|---------|----------|
| RANKL              | Abcam      | ab45039       |           | Mouse   | 1:200    |
| OPG                | Abcam      | ab9986        |           | Rabbit  | 1:500    |
| Integrin-\(β\)3   | Biorbyt    | orb248939     |           | Mouse   | 1:200    |
| RUNX2              | Abcam      | ab23981       |           | Rabbit  | 1:500    |
| Osteopontin        | Thermo Fisher | 14-9096-82  |           | Mouse   | 1:200    |
| Anti-mouse IgG1    | Molecular Probes | A21121   | Alexa 488 | Goat    | 1:200    |
| Anti-Rabbit IgG    | Molecular Probes | A21428   | Alexa 555 | Goat    | 1:200    |
| Anti-Rabbit IgG    | Molecular Probes | A21244   | Alexa 647 | Goat    | 1:200    |

Abbreviations: runt-related transcription factor 2 (RUNX2), receptor activator of nuclear factor κB ligand (RANKL), osteoprotegerin (OPG).
To explore the protein content of hPL, a total of 21 proteins that have been reported to influence bone resorption, formation or remodeling were quantified using multiplex immunoassays. In addition, calcium and phosphate concentration were quantified as well. The concentrations from these analyses were compared to effective concentrations from in vitro experiments reported in literature. The results from these quantifications and the literature research are reported in Table S2.

**Table S2.** Measured concentrations from hPL characterization experiments and effective concentrations reported in literature.

| Analyte | Effect on bone remodeling | Effective conc. from in vitro experiments | Conc. in hPL | Unit |
|---------|---------------------------|------------------------------------------|--------------|------|
| IL1-α   | Could enhance osteoclastic differentiation in the presence of RANKL (5,6)  
Suggested to enhance osteoclast survival (7)  
Can induce osteoblast apoptosis and inhibit osteogenesis (8) | 10,000  
NR  
500 – 10,000 | 283.94 | pg/ml |
| IL1-β   | Could enhance osteoclastic differentiation in the presence of RANKL (5,6)  
Could inhibit osteogenesis by decreasing RUNX2 expression, can stimulate ALP production and mineralization (9) | NR  
100 – 1,000 | 207.67 | pg/ml |
| IL4     | Can inhibit osteoclastic resorption in a dose dependent manner (10)  
Can inhibit osteoclast formation (11)  
Can inhibit osteogenesis in adipose tissue derived MSCs, which can be counteracted by IL6 (12,13)  
Can inhibit osteoblast proliferation and promote ALP production | 10,000 – 100,000  
100 – 10,000  
10,000  
100 – 10,000 | 32.72 | pg/ml |
| IL6     | Could enhance osteoclastic differentiation in co-culture by stimulating RANKL production by co-cultured cells (14) | NR | 623.59 | pg/ml |
| IL10   | Could suppress osteoclastic differentiation (17) | NR          | 196.80 | pg/ml |
|--------|------------------------------------------------|--------------|--------|-------|
|        | Can promote osteogenic differentiation of bone marrow derived MSCs at low physiological concentrations (18) | 10 – 1,000   |        |       |
|        | Can inhibit osteogenic differentiation of bone marrow derived MSCs at high pathological concentration (18) | 10,000 - 100,000 |        |       |
| IL17   | Different outcomes on osteoclasts reported (19) | NA          | 369.86 | pg/ml |
|        | Could directly induce osteoclastic differentiation (20) | 10 – 1,000   |        |       |
|        | Enhanced proliferation and stimulated osteogenesis. Can induce RANKL and M-CSF expression and osteoclastic differentiation in co-culture with PBMCs (21) | 20,000 – 50,000 |        |       |
| TNF-α  | Could enhance osteoclastic differentiation in the presence of RANKL (6) | NR          | 236.61 | pg/ml |
|        | In co- presence of IL-6, can induce osteoclastic differentiation in absence of RANKL (15) | 50,000       |        |       |
|        | Could inhibit osteogenesis by decreasing RUNX2 expression, can stimulate ALP production and mineralization (9) | 100 – 10,000 |        |       |
| Protein     | Function                                                                                     | Concentration | Unit |
|-------------|---------------------------------------------------------------------------------------------|---------------|------|
| SDF-1α      | Hypothesized to recruit osteoclast precursors (22)<br>Important role in migration of MSCs, especially in inflammation (23)<br>Could support early osteogenic differentiation (24) | ~8.526 – 255.8* | ng/ml |
| RANKL       | Expressed by a multiple cell types, but typically by osteoblastic cells, required for osteoclast differentiation (25). Multiple concentrations are used to induce osteoclastogenesis *in vitro* (26) | 10,000 – 100,000 | pg/ml |
| OPG         | Can prevent RANKL from binding to the RANK receptor on preosteoclasts, inhibits osteoclastogenesis (25,27) | 20 – 100       | ng/ml |
| Sclerostin  | Inhibits bone formation and osteogenesis and could stimulate RANKL secretion by osteocytes, thereby promoting osteoclastogenesis (28) | NR            | ng/ml |
| Osteopontin | Instrumental for intrafibrillar mineralization and promotes osteoclast activation (29)<br>Stimulates osteoclastogenesis and plays a major role in the formation of sealing zones (30)<br>Promotes osteoclast precursor migration (31) | 100,000**      | ng/ml |
| Dkk-1       | Inhibitor for osteogenic differentiation and bone formation. Can inhibit osteoclast induced mineralization by osteoblasts (32) | 200           | ng/ml |
| M-CSF       | Can regulate multiple steps of human *in vitro* osteoclastogenesis, including osteoclast precursor proliferation, differentiation, and fusion, and osteoclast resorption (33) | 25,000        | pg/ml |
| Growth Factor | Effect Description | Concentration | Unit |
|--------------|--------------------|---------------|------|
| GM-CSF       | Can suppress osteoclastogenesis in early differentiation stages, but promotes fusion of mature osteoclasts (34) | 3,000 | pg/ml |
| EGF          | Stimulatory effect on osteogenic differentiation of dental pulp stem cells, could enhance mineralization (35) | 10,000 | pg/ml |
|              | Could promote osteoblast proliferation and protein expression, but not mineralization (36) | 10,000 | pg/ml |
|              | Might improve osteoclast survival and differentiation through binding to the EFG receptor (37) | ≤ 10,000 | pg/ml |
|              | Could promote ALP production and mineralization by MSCs (38) | 1,000 – 10,000 | pg/ml |
| Basic FGF    | Inhibitory effect on osteogenic differentiation of dental pulp stem cells (35) | 10,000 | pg/ml |
|              | Might promote osteoblast-like cell differentiation towards osteocyte (39) | 10,000 | pg/ml |
|              | Contradictory results. Likely, proliferative and stemness maintaining effect at lower concentrations (40) | ≤ 10,000 | pg/ml |
|              | Could inhibit osteoclast formation when co-cultured with MSC-like cells (41) | 1,000 – 10,000 | pg/ml |
| VEGF         | Could enhance osteoclast survival and resorption (42,43) | 5,000 – 150,000 | pg/ml |
|              | Intracellular but not exogenous inhibits adipogenic differentiation and promotes osteogenic differentiation of bone marrow derived MSCs (43) | NA | pg/ml |
| Fetuin       | Could inhibit osteogenic differentiation and mineralization, could induce | 4,840,000,000* | ng/ml |
| Protein          | Effect                                                                 | Concentration       | Unit   |
|------------------|------------------------------------------------------------------------|---------------------|--------|
| **Fibronectin**  | Could inhibit osteoclastogenesis (46,47)                                | 0.1 – 20**          | µg/ml  |
|                  | Could enhance mature osteoclast activity and resorption (46)           | 20**                |        |
|                  | Could enhance osteogenic differentiation and bone-like matrix formation of bone marrow derived MSCs at low coating densities, and inhibit differentiation but promote proliferation at higher coating densities (48) | NA                  |        |
| **PDGF-BB**      | Can enhance osteoclastogenesis of macrophage-like cells (49)           | 20 - 50             | ng/ml  |
|                  | Could promote osteogenic differentiation of adipose derived but not bone marrow derived MSCs (50) | 20                  |        |
|                  | No effect on ALP formation and mineralization by MSCs (38)             | 10                  |        |
| **Calcium**      | Higher proteolytic activity in osteoclasts cultured with high calcium concentration than when cultured with a low concentration. Could improve attachment and migration with low calcium concentrations (51) | 1.2 (high)          | µmol/ml|
|                  | Too high calcium concentrations can inhibit osteoclast activity and from 20 µmol/ml induce osteoclast apoptosis (51,52) | 0.5 (low)           |        |
|                  | Could promote proliferation and osteogenic differentiation of MSCs (53) | 5 - 20              |        |
|                  |                                                                        | 7.8                 |        |
### Phosphate

| Effect                                                                 | Concentration | Calculated (µmol/ml) |
|------------------------------------------------------------------------|---------------|----------------------|
| Inhibited osteoclastogenesis from bone marrow cultures in co-culture with osteoblast-like cells (54) | 1 – 10        | 1.948                |
| Inhibited osteoclastogenesis of human PBMCs and macrophage-like cells in a dose-dependent response (55) | 1.5 – 4       |                      |
| Can promote proliferation of human bone marrow derived MSCs (56)        | 2 – 10        |                      |
| Can promote migration, osteogenic differentiation and mineralization of human bone marrow derived MSCs (56) | 4 – 10        |                      |

* Calculated from the molecular weight found in literature

** Concentration used for coating of culture substrate

Abbreviations: human platelet lysate (hPL), concentration (conc.), not reported (NR), not applicable (NA), monocyte (MC), mesenchymal stromal cell (MSC), peripheral blood mononuclear cell (PBMC), runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), interleukin (IL), tumor necrosis factor (TNF), stromal derived factor (SDF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor κB ligand (RANKL), osteoprotegerin (OPG), Dickkopf WNT Signaling Pathway Inhibitor 1 (Dkk-1), epidermal growth factor (EGF), basic fibroblastic growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet derived growth factor-BB (PDGF-BB).
3 Supplementary Figures

To visualize the non-resorbed surface, osteo assay wells were stained with a modified Von Kossa. To capture the entire well, tile scans were made with a bright field microscope. Tile scans were stitched with Zen Blue software (version 3.1, Zeiss, Breda, The Netherlands). To enable segmentation and resorption quantification, scratches that were introduced by mechanical cell removal in co-cultures were manually masked whereafter image contrast was increased using Fiji (4). A clipping mask was created in Illustrator (Adobe Inc., San Jose, CA, USA) to remove the edges of the wells. Segmentation was performed in MATLAB (version 2019b, The MathWorks Inc., Natrick, MA, USA), using Otsu’s method for binarization with global thresholding, where the threshold was kept constant throughout the entire image (Figure S1) (57).

![Workflow osteo assay wells from raw data to image segmentation. Decellularized resorption wells (A) were stained with Von Kossa (B). Scratches were manually masked (C) and a clipping mask was used to remove the edges of the well (D). Lastly, images were segmented such that the resorbed surface could be quantified (E).](image_url)
As osteoclasts have a life-span of approximately 2-3 weeks \((58,59)\), culture photographs were taken on day 18/21 of MC mono-cultures (Figure S2). By day 18, MCs have likely differentiated into mature osteoclasts and apoptosis might not yet have taken place.

**Figure S2.** Micrographs of all MC donors on day 18 in mono-cultures. Clear differences can be observed between MCs cultured with FBS and hPL, indicating a heterogeneous cell population in MCs cultured with FBS. No clear differences were observed between donors, with only in donor 1 a concentration dependent size difference in MCs cultured with hPL. Abbreviations: fetal bovine serum (FBS), human platelet lysate (hPL), monocyte (MC).
To check whether osteogenically stimulated MC-MSC co-cultures showed differences in RANKL and OPG expression, cells were stained for these proteins (Figure S3).

**Figure S3.** Micrographs of osteogenically stimulated MC-MSC co-cultures, stained for F-Actin (red), the nucleus (gray), OPG (blue) and RANKL (green). It is expected that OPG and RANKL also played a role in the inhibition of osteoclastic differentiation in osteogenically stimulated MC-MSC co-cultures, although no clear differences between groups were found after immunocytochemical staining of OPG and RANKL. Scale bar in insert is 20 µm. Abbreviations: fetal bovine serum (FBS), human platelet lysate (hPL), monocyte (MC), mesenchymal stromal cell (MSC), receptor activator of nuclear factor κB ligand (RANKL), osteoprotegerin (OPG).
3D osteogenically stimulated MSC mono-culture

A

Collagen

B

Osteogenesis

C

Mineralization

D

DNA

DNA content [ng/μg scaffold]

10% FBS 10% hPL 5% hPL 2.5% hPL

E

ALP in cell lysate

ALP activity [nmol/mg/min]

10% FBS 10% hPL 5% hPL 2.5% hPL

F

OPG in supernatant

OPG [pg/ml]

10% FBS 10% hPL 5% hPL 2.5% hPL

G

RANKL in supernatant

RANKL [pg/ml]

10% FBS 10% hPL 5% hPL 2.5% hPL
Figure S4. Results from 3D osteogenically stimulated MSC mono-cultures indicating most osteogenic differentiation and bone-like matrix formation in MSCs cultured with FBS. (A) Picrosirius red staining indicating collagen formation in osteogenically stimulated 3D MSC monocultures after 4 weeks culture, most collagen seemed present in constructs cultured with 10% FBS and 10% hPL. (B) Staining for osteogenic markers indicating osteogenic differentiation of 3D MSC monocultures after 4 weeks culture in all conditions. Sections were stained for cell nuclei (gray), collagen (red), osteopontin (green) and RUNX2 (blue). (C) Micro-computed tomography scans of constructs, indicating most mineralization in constructs cultured with FBS. No mineralization was found in constructs cultured with 2.5% hPL. (D) DNA quantification in cultured constructs, no clear differences were found between different conditions. (E) ALP activity quantification revealed most ALP in lysates of constructs cultured with 10% FBS, \( p<0.05 \) (one-way ANOVA and Turkey’s post hoc tests) (F) OPG quantification in cell supernatants of 2 bioreactors (containing 4 tissue constructs each) revealed most OPG in constructs cultured with 10% FBS. (G) RANKL was only detected in constructs cultured with 10% hPL. (*\( p<0.05 \), **\( p<0.01 \)) Abbreviations: fetal bovine serum (FBS), human platelet lysate (hPL), monocytes (MCs), mesenchymal stromal cells (MSCs), runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), osteoprotegerin (OPG), receptor activator of nuclear factor kB ligand (RANKL), not detected (ND).
4 Supplementary References

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