**Electronic Supplementary Material**

# Bioactivity of surface tethered Osteogenic Growth Peptide motifs

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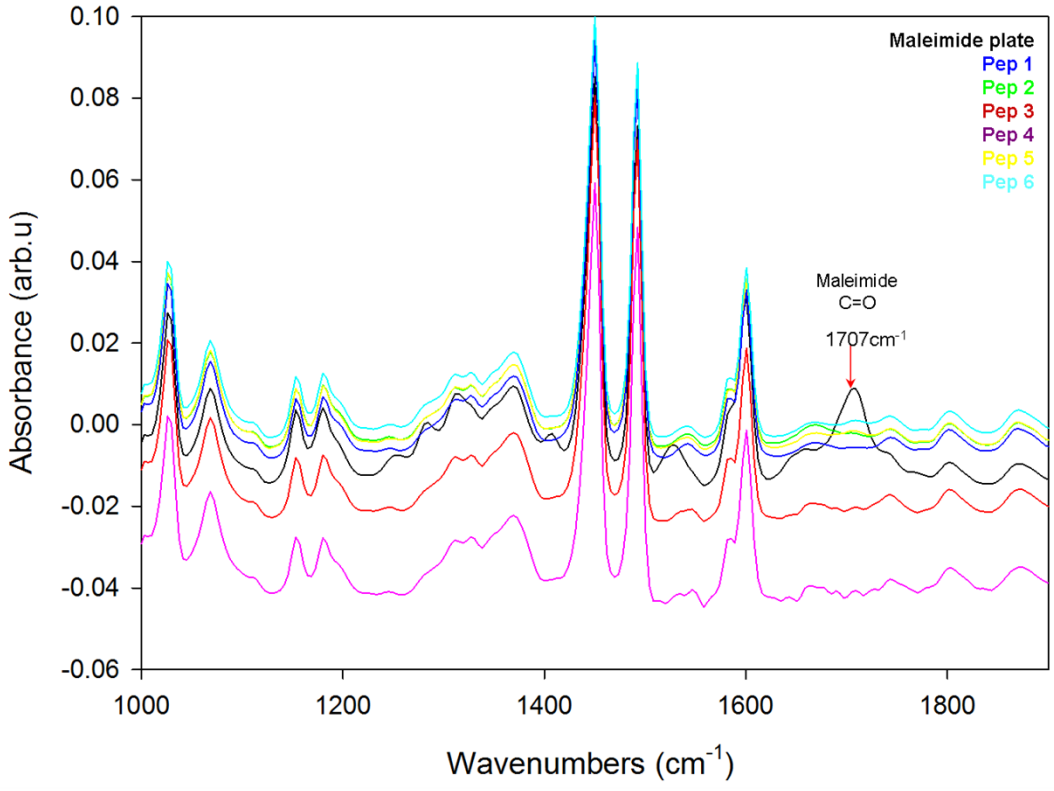
## Experimental procedures

All peptides were purchased from Proteogenix, Oberhausbergen, France.

### OGP motifs tethering to SULFHYDRYL-BIND™ polystyrene plates

According to manufacturer protocol (*Corning's Sulfhydryl-BIND*™, Corning, NY), plates were incubated for 1 h at RT with 1 mM solution (MilliQ™ water) of each peptide (Pep 1-6). After the coupling, the plate was thoroughly washed with PBS buffer solution. The effectiveness of the coupling was determined by FT-IR:



**Mesenchymal stem cell culture**

Rabbit mesenchymal stem cells were isolated from the rabbit bone marrow and cultured in α-MEM medium plus 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (100 U/ml - 100 µg/ml). MSCs at P3 were plated at a density of $5.0\times10^3$ cells/cm$^2$ in 96-well plates and cultured in presence of $0.05x10^{-6}$ mmol grafted peptide sequences (Pep 1, Pep 2, Pep 3, Pep 4, Pep 5 and Pep 6 group) or not-immobilized peptide sequences (Pep 7, Pep 8 and Pep 9 group).
Moreover a group of cells cultured directly on tissue culture plastic was used as control (cells only group). Cells were incubated for 1, 3, 7 and 14 days. Culture media, without any osteogenic supplements, was changed every three days, with reposition of the peptide levels for Pep 7, Pep 8 and Pep 9 groups. All cell culture was performed at 37°C in an atmosphere of 5% $CO_2$ and all cell handling procedures were performed in a sterile laminar flow hood.

**Cell proliferation assay**

The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen) was prepared at 5 mg/ml in 1x PBS. Cell were incubated with the MTT reagent 1:10 for 2 h at 37°C. Medium was collected and cells incubated with 1 ml of Dimethyl sulfoxide (Sigma) for 15 min. In this assay, the metabolically active cells react with the tetrazolium salt in the MTT reagent to produce a formazan dye that can be observed at λmax of 570 nm, using a Multiskan FC Microplate Photometer (Thermo Scientific). This absorbance is directly proportional to the number of metabolically active cells. Mean values of absorbance were determined. Five samples were analysed per time point.

**Alkaline Phosphatase (AP) assay**

Cell AP activity was quantified using an enzymatic assay based on the hydrolysis of *p*-nitrophenyl phosphate (pNP-$PO_4$) to *p*-nitrophenol (pNP)[1]. Briefly, 50 µl of cell lysate, obtained after cell incubation with 100 µl 1x PBS with 0.1% (v/v) Triton-X, was added to pNP-$PO_4$ solution (Sigma-Aldrich) and allowed to react at 37 °C. Absorbance was read at 0, 30, 60 and 90 min at λmax of 405 nm, using a microplate reader (Tecan, Research Triangle Park, NC) and AP activity calculated by cross-reference to a standard curve of nanomoles of

p-nitrophenol liberated per cell. AP activity was normalized to total cell number, as measured by the MTT assay, and to the control group. Three samples were analysed per condition at day 7.

**Immunofluorescence and Hematoxylin and eosin (H&E) staining**

Groups Pep 1, Pep 5, Pep 9 and cells only were subjected to FITC-conjugated phalloidin immunocytochemistry and H&E analysis. Detailed methods can be found in ESI.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Gene expression was quantified for Runt-related transcription factor 2 (RUNX2), Bone Morphogenetic protein 2 (BMP2) and Osteonectin (SPARC) on Groups Pep 1, Pep 5, Pep 9 and cells only, at day 7 and 14.

At day 7 and 14, cells grown in presence of peptides 1, 5, 9 and the CT group were collected by trypsinization, centrifuged, resuspended in RNAlater solution and kept at -20ºC.Total RNA extraction was performed by use of the Tris reagent, followed by the Purelink RNA Mini kit according to manufacturer's instructions. In detail, we have used a on-column DNAse treatment by use of the Purelink DNAse set. Purified total RNA was eluted with THE RNA storage solution and kept at -80°C until reverse transcription. RNA integrity was analysed by native agarose gel electrophoresis and quantification performed by the Qubit® 2.0 Fluorometer together with the Qubit® RNA BR assay kit, following manufacturer's instructions. Total RNA was reverse transcribed to cDNA (RT) using the High-Capacity cDNA Reverse Transcription Kit, according to manufacturer's instructions. In detail, we have used 1.5 µg of total RNA in a 20 µl final reaction volume for each sample, as follows: 2 µl of 10x RT buffer, 0.8 µl of 25x dNTP mix, 2 µl of 10x RT primers, 1 µl of RNAse inhibitor, 3.2 µl of Nuclease free water plus 10 µl of template. Reaction conditions: 25ºC for 10 min, 37ºC for 120 min, 85ºC for 5 min. cDNA was kept at -20ºC until qPCR.

Quantification of gene expression for Runt-related transcription factor 2 (RUNX2), Bone Morphogenetic protein 2 (BMP2), Osteonectin (SPARC) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, used as housekeeping gene) was performed by use of the StepOne™ Real-Time PCR System (Applied Biosystems). The following

Taqman gene expression mastermix and the following Taqman gene expression assays: RUNX2: Oc02386741_m1, BMP2: Oc03824113_s1, SPARC: Oc03395842_m1 and GAPDH Oc03823402_g1, all with the Fam™ dye as reporter.
1 µl of cDNA was used in a 10 µl final reaction volume, as follows: 5 µl of 2x mastermix, 0.5 µl of 20x gene expression assay, 3.5 µl of Nuclease free water plus 1 µl of template. qPCR reaction was performed on a 48-well plate adhesive sealed, as follows: 40 cycles at 95ºC for 15 sec, 60ºC for 1 min, after an initial denaturation step of 50ºC for 2 min and 95ºC for 10 min. Experiments was done in triplicate, using three tecnical replicates for each experiment. Data was collected using the OneStep Software (v.2.2) and relative quantification was performed using the comparative threshold (Ct) method (ΔΔCt), where relative gene expression level equals $2^{-\Delta\Delta Ct}$ [20], as described by the manufacturer.
In detail, changes in gene expression level of the target genes RUNX2, BMP2 and SPARC were calculated by normalization to the reference GAPDH and by normalization to the control condition, used as calibrator, within each sample set. StepOne™ Real-Time PCR System was used and all equipment and reagents were from Life Technologies.

**Statistical Analysis**

Results were expressed as MEAN±SEM plotted on graph. Analysis of the effect of the peptide sequences on cell culture was made by one- or two-way ANOVA, followed by Bonferroni's post-hoc test. Statistical analyses were performed by the GraphPad Prism software (version 5.0), with statistical significance set at $p \leq 0.05$.

**Phalloidin immunofluorescence staining.** Cells were washed with 1x PBS for 5 min, fixed with 4% (w/v) paraformaldehyde for 15 min and washed with 1x PBS for 5 min. Permeabilization was performed with 1x PBS with 0.1% (v/v) Triton X-100 for 5 min. FITC-conjugated Phalloidin antibody (Invitrogen) 1:500 in 1x PBS was added for 20 min at room temperature in the dark. Cells were washed with 1x PBS for 5 min and incubated with DAPI (Invitrogen) in 1x PBS for 5 min. Images were acquired by

an Inverted Ti-E fluorescence microscope (Nikon). One sample (Pep 1, Pep 5, Pep 9 and cells only as control group) per time point was analysed.

**Hematoxylin and eosin (H&E) staining.** Cells were washed with 1x PBS for 5 min, fixed with 4% (w/v) formaldehyde for 15 min, washed twice with 1x PBS for 5 min and stained with hematoxylin and eosin.

Briefly, the cells were incubated in Mayer's hematoxylin for 10 min and differentiated with tap water for 10 min. Cells were, finally, stained with eosin Y (0.25%) 1-2 min, washed with distilled water and mounted. Images were acquired by an Inverted Ti-E fluorescence microscope (Nikon). One sample (Pep 1, Pep 5, Pep 9 and cells only as control group) per time point was analysed.

[1] C. C. Teixeira, M. Hatori, P. S. Leboy, M. Pacifici and I. M. Shapiro, *Calcif Tissue Int*, 1995, **56**, 252-256.