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Directing hMSCs Fate through Geometrical Cues and Mimetics Peptides

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For Peer

1
2 ABSTRACT
3

4 The native microenvironment of mesenchymal stem cells (hMSCs) - the
5 extracellular matrix (ECM), is a complex and heterogenous environment
6 structured at different scales. The present study aims at mimicking
7 the hierarchical microorganization of proteins or growth factors within
8 the ECM using the photolithography technique. Polyethylene
9 terephthalate (PET) substrates were used as a model material to
10 geometrically defined regions of RGD + BMP-2 or RDG + OGP mimetic
11 peptides. These ECM-derived ligands are under research for regulation
12 of mesenchymal stem cells osteogenic differentiation in a synergic
13 manner. The hMSCs osteogenic differentiation was significantly
14 affected by the spatial distribution of dually grafted peptides on
15 surfaces, and hMSCs cells reacted differently according to the shape
16 and size of peptide micropatterns. Our study demonstrates the presence
17 of a strong interplay between peptide geometric cues and stem cell
18 differentiation toward the osteoblastic lineage. These tethered
19 surfaces provide valuable tools to investigate stem cell fate
20 mechanisms regulated by multiple ECM cues, thereby contributing to the
21 design of new biomaterials and improving hMSCs differentiation cues.
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50 1. Introduction

51 In the field of bone tissue engineering, mesenchymal stem cells (MSCs)
52 are considered as good potential candidates due to their high
53 proliferation rate, multipotency and bioavailability.(1) One of the
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1
2 hypotheses is that, MSCs travel from their niche to the needed
3
4 site to repair the injured tissues and restore their functions.(2) The
5
6 stem cell niche is a highly structured and complex microenvironment
7
8 where the stem cell renewal and differentiation take place.(3) The key
9
10 component of these microenvironments is the extracellular matrix (ECM).
11
12 The ECM influences the MSCs fate through various stimuli, which can be
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14 biological, chemical or even mechanical. Though, the cell response
15
16 depends on the abundance and distribution of the biochemical molecules
17
18 in the ECM of the stem cell niche.(4) For example, during the MSCs
19
20 proliferation phase, the native ECM has a higher concentration in
21
22 fibroblast growth factor-2 (5) while, during the osteogenic
23
24 differentiation, the ECM is richer in bone morphogenic protein-2 (BMP-
25
26 2) and organization of the ECM undergoes a remodeling.(6)

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29 Based on this knowledge, many strategies to translate the native ECM
30
31 features to *in vitro* models used these growth factors to control the
32
33 MSCs fate.(7,8) A traditional approach consists in the immobilization
34
35 of these biochemical cues onto the surface of bioinert materials in
36
37 order to mimic physiological conditions.(9) Moreover, coatings of
38
39 adhesion proteins and growth factors onto materials have been used
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41 since a combination effect, regulating osteogenesis among others. It
42
43 is now demonstrated that integrins plays a key role in osteogenesis
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45 while located nearby growth factors receptors.(10-13) Albeit,
46
47 researchers are trying to create the ideal biomaterial through surface
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49 modification in order to satisfy the properties of the native ECM.
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2 A promising way for the development of ideal biomaterials involves a
3
4 certain level of attention to the spatial arrangement of the native
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6 ECM, which has been identified as a trigger during the stem cell
7
8 differentiation.(7,14-19) As a matter of fact, during proliferation
9
10 and differentiation, stem cells encounter a temporally and spatially
11
12 controlled mix of biochemical cues.(20,21) This knowledge is supported
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14
15 by various *in vitro* studies which highlight the fact that very distinct
16
17 cellular responses can be obtained through the spatial organization of
18
19 ECM biomolecules.(16) Thus, biomolecules patterning of adhesion
20
21 molecules and growth factors could be the next step toward the
22
23 elaboration of biomaterials to mimic the native ECM *in vitro*.
24

25
26
27 By transferring the recent developments in microengineering technology
28
29 to surface modification, the patterning of biomolecules onto the
30
31 surface of a biomaterial can now be performed. The field of
32
33
34 biomaterials has been extensively using microfabrication techniques to
35
36 replicate the complexity of the native ECM.(22) The study presented
37
38 herein describes a technique to spatially pattern two mimetic peptides
39
40 onto a model material, polyethylene terephthalate (PET) films. The
41
42 peptides used in this study are RGD, a cell adhesion promoter, and
43
44
45 BMP-2 or OGP₁₀₋₁₄ (osteogenic growth peptide), to induce stem cell
46
47 osteogenic differentiation.(13,23,24) The RGD sequence and BMP-2
48
49
50 mimetic peptides are known to act synergistically to promote osteogenic
51
52 differentiation when randomly co-tethered onto a biomaterial
53
54 surface.(18,23,25) Spatial organization of ECM biomolecules has
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56
57 already been used to control MSCs fate, but with confined single cells
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2 (26,27) or with different cell type.(28,29) Moreover, most of these
3
4 studies were focusing on the impact of organized tethered ligands on
5
6 cellular adhesion. Our approach combined a cell adhesion promoter -
7
8 the RGD peptide - and peptides known to induce osteogenic
9
10 differentiation - the BMP-2 mimetic peptide and OGP₁₀₋₁₄. These peptides
11
12 were combined according to different shapes (squares, rectangles,
13
14 hexagons) or different square sizes at the micrometric scale to control
15
16 the cell adhesion while promoting the osteogenic differentiation, to
17
18 closely mimic the native ECM and gather information about the stem cell
19
20 interaction with their microenvironment.
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23 24 25 2. Materials and Methods

26 27 2.1. Materials

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29 PET samples were taken from a commercial crystalline biaxially-oriented
30
31 film obtained from GOODFELLOW (LILLE, France). The bi-oriented film had a
32
33 thickness of 75 μm . Inorganic reagents (NaOH, KMnO_4 , H_2SO_4 , HCl, glacial
34
35 acetic acid), acetone, acetonitrile, dimethylaminopropyl-3-
36
37 ethylcarbodiimideethylcarbodiimide hydrochloride (EDC), N-
38
39 hydroxysuccinimide (NHS) and 2-(N-morpholino)-ethanesulfonic acid
40
41 (MES), and toluidine blue-O (TBO) were purchased from SIGMA-ALDRICH (LYON,
42
43 France). GRGDSPC (RGD), GYGFGG (OGP), RKIPKASSVPTLSAISMLYL, which is
44
45 a BMP-2 mimetic peptide previously identified by our group (BMP-2)
46
47 (18,19,30), GRGDSPC-TAMRA, and RKIPKASSVPTLSAISMLYL-FITC fluorescent
48
49 peptides were synthesized by GENECUST, (ELLANGE, Luxembourg).
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2.2.1. Surface preparation of PET and covalent grafting of the different peptides

PET surfaces were modified according to Chollet *et al.* (31) with some modifications. Briefly, PET was hydrolyzed and oxidized in order to create carboxyl groups on the surface (labeled as ``PET-COOH``). Then, the surfaces were immersed in a solution of EDC (0.2 M) + NHS (0.1 M) + MES (0.1 M) in MilliQ water for activation.

2.2.2. Preparation of resist patterned surfaces

Resist patterns were created on glass substrates using photolithography. Briefly, photosensitive resist S1818 (CHIMIE TECH, France) was coated on glass surfaces and spun at 3000 rpm for 30 s, leading to a homogenous photoresist of approximately 1 μm . The surfaces were then baked at 100 °C for 60 s prior exposure to a pattern of light emitted by UV lamp (365 nm, 19,5 mW/cm², contact mode, 50 Hz, exposure time: 8 s) through photomasks with patterns of different geometries (see Figure 1) (Département de génie électrique et de génie informatique, Université de Sherbrooke, QC, Canada). Subsequently, the exposed resist was developed by immersing the substrates in Microposit Developer solution (MF319, CHIMIE TECH, France) for 40 s. Finally, the samples were washed with deionized water, to remove any traces of developed resist, and dried with nitrogen gas (Figure 1).

2.2.3. Peptide grafting and patterning

The covalent grafting of peptides was achieved as described in a previous publication.(32) Briefly, the activation step was followed by creating resist patterns on activated surfaces using photolithography

1
2 as described in the previous paragraph. Finally, resist patterned
3
4 surfaces were immersed in peptide solution (peptides dissolved in PBS
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6 at the concentration of 10^{-5} M) for 16h at room temperature. After
7
8 reaction, samples were washed with deionized water under agitation,
9
10 and then immersed in acetone for 30 seconds to remove the resist
11
12 pattern, resulting in peptide patterns surrounded with activated
13
14 domains. Then the second peptide was grafted following the same
15
16 protocol. Finally, substrates were rinsed and sonicated with MilliQ
17
18 water (Figure 2). Patterns of RGD-TAMRA and BMP-2-FITC or OGP-FITC
19
20 peptides developed using this protocol were shaped as hexagons, squares
21
22 or rectangles (Figure 1). Unpatterned and unfunctionalized PET surfaces
23
24 functionalized were also prepared and used as controls for biological
25
26 experiments.
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30 31 32 2.2.4. Surface characterization

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34 The covalent grafting of peptides, the density of grafted peptides as
35
36 well as the surface roughness after each step of surface modification
37
38 were evaluated in a previous work on unpatterned PET surfaces using X-
39
40 ray photoelectron spectroscopy, fluorescence microscopy, contact
41
42 angle, and atomic force microscopy (32). In the present work, we have
43
44 focused on evaluating the efficiency of peptide patterning using
45
46 fluorescence microscopy and optical interferometry. On resist
47
48 patterned surfaces, fluorescence microscopy (LEICA DM5500B, WETZLAR,
49
50 Germany) was used to characterize the shape of resist patterns while
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52 optical interferometry (Bruker Nano-NT9080, KARLSRUHE, Germany) was
53
54 employed to measure the pattern dimensions. Resist patterns were
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1
2 visible under fluorescence because the S1818 resist is auto-fluorescent
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4 when excited with a 543nm laser line. Optical interferometry
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6 measurements were carried out on dry samples, at room temperature,
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8 using the vertical scanning interferometry mode with a vertical
9
10 resolution of approximately 2 nm. The interferograms were digitalized
11
12 with a CCD camera and converted into 2D topographic maps. Pattern
13
14 dimensions, according to the X and Y axes, were measured on these maps
15
16 using Veeco software.

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21 These PET surfaces containing resist patterns were then used as a
22
23 template for fluorescent RGD and BMP-2 or OGP patterning. Finally, the
24
25 spatial distribution of peptides was visualized under fluorescence
26
27 microscopy (Leica microsystem DM5500B, microscope with a motorized,
28
29 programmable stage using a CoolSnap HQ camera controlled by Metamorph
30
31 7.6).

32 33 34 35 2.2.5. Cell culture

36
37 Human mesenchymal stem cells (hMSCs) from bone marrow (one donor)
38
39 purchased from PromoCell (Heidelberg, Germany), were grown in
40
41 mesenchymal stem cell basal media (MSCBM2) (PromoCell) in a humidified
42
43 atmosphere containing 5% (vol/vol) CO₂ at 37 °C. For each experiment,
44
45 hMSCs between passages 4 and 5 were seeded on PET materials at at an
46
47 identical density of 5,000 cells/cm² for all materials in serum-free
48
49 α-MEM during the first 6 h. The medium was then changed to α-MEM
50
51 supplemented with 10% (v/v) fetal bovine serum FBS (Gibco) with no
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53 additional growth factors and was changed every 72 h. hMSC
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1
2 differentiation on the different PET substrates was evaluated after
3
4 2 weeks of cell culture. Due to the large number of materials (more
5
6 than 140 materials) required to perform the biological analyses, the
7
8 decision was made to perform cell cultures at one specific time point.
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11 2.2.6. RT Quantitative Real-Time PCR

12 hMSCs were lysed in TRIZOL reagent (Invitrogen) to isolate the total
13
14 RNA. TurboDNA free kit (Ambion) was used to remove contaminating DNA
15
16 from RNA preparations. 2 µg of purified total RNA were used to
17
18 synthesize cDNA using Thermo Scientific Maxima Reverse Transcriptase
19
20 (Thermo Scientific) and random primers (Thermo Scientific). cDNA
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22 aliquots (4 ng) were then amplified in 10 µL reaction volume containing
23
24 500 nM primers and SsoAdvanced™ Universal SYBR® Green Supermix
25
26 (Biorad) using CFX96™ Real-Time PCR Detection System (Biorad). PCR
27
28 cycling parameters were as follow: denaturation at 95 °C for 30 s
29
30 followed by 40 cycles of PCR reactions (95 °C for 5 s and 60 °C for 10
31
32 sec). Cq values for the gene of interest were normalized against two
33
34 genes: RPC53 and PPIA. Bestkeeper software was used to determine
35
36 normalization effectiveness of each reference gene among all samples.
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38 The relative expression levels were calculated using the comparative
39
40 method (2-ΔΔCt) and controls were arbitrarily set at 1. Primers used
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42 for amplification are listed in Table 1.
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48 2.2.7 Statistical Analyses

49 All data were expressed as the mean ± standard deviation (SD) and
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51 analyzed by one-way analysis of variance (ANOVA) and Tukey's test for
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53 multiple comparisons, using GraphPad Prism version 6.01 for Windows
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2 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Significant
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4 differences were determined for p values of at least ≤ 0.05 . * $p \leq$
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6 0.05 , ** $p \leq 0.01$, and *** $p \leq 0.001$.

7 8 9 3. Results

10 11 3.1. Characterization of patterned surfaces

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13 The grafting protocol to conjugate peptides on the patterned surfaces
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15 was previously described.⁽¹⁸⁾ Photolithography was used on activated
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17 surface (PET-NHS) to create resist patterns. All the surfaces with the
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19 resist patterns were assessed under fluorescence microscopy due to the
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21 S1818 resist auto-fluorescence. Images clearly showed the precise
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23 geometries shaped as hexagons, rectangles and squares (Figure 3). After
24
25 the qualitative assessment of the resist patterned surfaces, the
26
27 quantitative assessment of these surfaces was performed using optical
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29 interferometry. The obtained surface profiles revealed that resist
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31 pattern sizes are closed to the originally defined micro-sized features
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33 (Table 2 and Figure 3).

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39 Then, the first fluorescent peptide to be grafted (RGD-TAMRA) was
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41 putted into contact with the resist micropatterned surfaces. Therefore,
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43 only the available area was grafted with the first peptide. After
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45 removing the resist, the surfaces were placed into a solution of the
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47 second peptide to graft (BMP-2-FITC or OGP-FITC). Fluorescence
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49 microscopy confirmed the efficiency of peptide patterning. Images
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51 showed identifiable patterns and exhibit the expected shapes
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53 (hexagonal, squared and rectangular geometries) and size (Figure 4),
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2 and the intensity profile exhibits no overlap of the different regions
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4 (Figure 5). Surfaces for cell culture were produce in the exact same
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6 way, from the same batch of materials.
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8 9 3.2. hMSCs osteogenic differentiation

10 First, it is worth mentioning that a previous work investigated the
11 effect of the homogeneous conjugation of individual peptides (RGD, BMP-
12 2, and OGP) (32). With few exceptions, these single-tethered peptide
13 surfaces exhibited lower expressions for all three markers (RUNX2,
14 collagen I α -1, and OCN) investigated in the present study, therefore
15
16 pointing to a synergistic effect toward cell differentiation when the
17 so-called adhesion and differentiation signal peptides were co-
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19 conjugated on a surface. Accordingly, any additional marker expression
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21 response measured while comparing homogeneous surface conjugation of
22 the adhesion peptide (RGD) together with a differentiation peptide
23 (BMP-2 or OGP) can only be attributed to pattern shapes or sizes.
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36 37 3.2.1. The extent of hMSCs osteogenic differentiation in response to 38 different shapes of patterns

39 The potential changes in hMSCs phenotype on the different shapes of
40 patterned surfaces were assessed by RT-qPCR after 2 weeks of cell
41 culture. Human MSCs seeded on oxidized PET in the same cell culture
42 conditions were used as negative control. Surfaces conjugated with a
43 mixture of RGD and BMP-2 peptides were first investigated. At first
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45 sight, the results showed that the expression of RUNX2, an early
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47 osteogenic marker, was significantly enhanced in the cells cultured on
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49 the tethered surfaces, as compared to control surfaces (Figure 6A). On
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1
2 the other and, the organization of RGD and BMP-2 peptides as squares,
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4 hexagons, or rectangles did not lead to additional RUNX2 expression
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6
7 as compared to the surface randomly conjugated with the mixture of
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9 peptides. Similar trends were observed while considering ColI- α 1
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11 expression, with significant differences recorded when RGD and BMP-2
12
13 are patterned as rectangles and hexagons as compared to the control
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15 sample or to the surface homogeneously coated with both peptides
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17 (Figure 6B). Finally, the OCN expression did not allowed to
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19 discriminate among all investigated samples, likely because that the
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21 culture time that was investigated in the present study was not long
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23 enough to allow measuring differences in this marker expression (Figure
24
25 6 C).

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29 Surfaces with a mixture of RGD and OGP were also investigated.
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31 In this case, the situation is less clear in terms of RUNX2
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33 expression. Indeed, the presence of both RGD and OGP on the various
34
35 investigated samples clearly lead to an increase of the RUNX2
36
37 expression as compared to the control sample (Figure 6D). However, no
38
39 significant differences were evidenced among the various investigated
40
41 patterns. That said, the expression of ColI- α 1 shed more light on the
42
43 effect of the RGD and OGP organization on the surfaces as the squares
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45 definitely lead to a significant increase of this marker with respect
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47 to all other investigated samples (Figure 6E).
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49 Again, it sounds that the culture time was not long enough
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51 to enable measuring differences in the OCN expression, therefore
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1
2 providing an indication about the state of the differentiation level

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4 (Figure 6F).

5 Taken together, the data on both

23
24 the RGD/BMP-2 and RGD/OGP surface patterning point toward an improved

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26 cell differentiation related to the peptide couple organization on the

27
28 surface. In addition, it is also clear that an identical geometrical

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31 organization of the RDG/BMP-2 and RGD/OGP couples was not felt

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33 similarly by the hMSCs in terms of their differentiation.1.

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45 3.2.2. The extent of hMSCs osteogenic differentiation in response to
46 different size of patterns

47 The potential changes in hMSCs phenotype on the surfaces patterned with
48 different sizes of squares were assessed by RT-qPCR after 2 weeks of
49 cell culture (Figure 7). Human MSCs seeded on oxidized PET in the same
50 cell culture conditions were used as negative control. Surfaces with
51 a mixture of RGD and BMP-2 peptides were first investigated. With the
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exception of the 100x100 sample, the expression of RUNX2 (Figure 7A) is more important on the RGD/BMP-2 tethered surfaces (random, 50x50 and 25x25) as compared with the control sample, with the most important expression being observed on the smallest size of square pattern. Of note, a nice gradation of the RUNX2 expression was observed from the larger (100x100) to the smaller (25x25) RGD/BMP-2 tethered surfaces. For the RGD/BMP-2 couple, the importance of the pattern size on the cell differentiation behavior was also observed while measuring the collagen I α -1 and OCN expression (Figure 7B and 7C), as both markers exhibited the highest measured level among all investigated samples when cells were cultured on the 25x25 pattern.

The situation was somewhat different while investigating the effect of peptide pattern size with the RGD/OGP couple. On one hand, the random, 100x100, and 50x50 samples all led to an almost equivalent four-fold increase of the RUNX2 expression (Figure 7D) as compared to the control sample. On the other hand, the expression of RUNX2 was again higher on the smaller size pattern, that is the 25x25 sample. In this case, this marker level of expression was eight times that of the control sample and twice that of any other investigated RGD/OGP conjugated surfaces, either homogeneously conjugated or patterned. For this peptide couple, the expression of ColI- α 1 (Figure 7E) was significantly higher only in the case of the medium size (50x50) and OCN (Figure 7F) was not significantly impacted by the tethered peptides in any cases.

34. Discussion

Promoting a specific fate of hMSCs is a complex process involving different parameters such as cell morphology, gene expression and ECM protein concentration changes. *In vivo*, the osteogenic differentiation process is ruled through different stimuli, which can be chemical and/or physical in nature.(3,4) The differentiation of hMSCs in the stem cell niche is guided by those stimuli. *In vitro*, scientists are trying to reproduce the stem cell niche to promote stemness or induce a guided differentiation. The use of peptide appears to be a good solution to mimic the stem cell niche. For example, BMP-2 mimetic peptides were used *in vitro*,(18,23,33) in animal models (34) and are FDA approved for various surgeries such as spinal cord fusion procedure.(35,36) However, to overcome the diffusion of the mimetic peptide away from the implant when placed in the body, immobilization on biologically compatible biomaterials surface can be used.(37)

Osteogenesis is induced through the interaction of BMP-2 growth factor with its receptor - BMP transmembrane receptors type I and type II (BMPR-I and BMPR-II). BMP-2 preferentially interact with BMPR-II which activate the phosphorylation of SMAD1/5/8 and their translocation into the nucleus. Following the translocation, RUNX2 expression is significantly improved as another early markers regulating osteoblast differentiation.(38) The expression of RUNX2 further activates the upregulation of osteoblast phenotype proteins.(39) Although BMP-2 peptide is greatly used in biomaterial strategies to promote bone regeneration, current strategies for the design of biomaterials for

1
2 bone tissue engineering are using multiple peptides combined on the
3
4 surface of a biomaterial. (18,19,23) This strategy is used to mimic the
5
6 physiological situation, where a combinatorial effect of a mixture of
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8 ligands synergize together to induce the differentiation process.
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10 Ligand crosstalk has been investigated in order to differentiate stem
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12 cells. For example, various combinations of BMP-2 (sequence used
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14 KIPKASSVPTELSAISTLYL), osteopontine (OPN) and RGD were used to induce
15
16 the differentiation of rat MSCs cells. (40) An increase of ALP activity
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18 and calcium content after 2 and 4 weeks on the hydrogels containing
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20 RGD alone, and even further with the hydrogels containing the mixture
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22 of RGD and BMP-2 and/or OPN. We recently demonstrated that the
23
24 expression of alkaline phosphatase is more important on surfaces
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26 tethered with FHRRIKA and BMP-2 peptides together, without requiring
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28 differentiation media during the cell culture. (32)
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34 Another growth factor which can induce hMSCs differentiation is
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36 osteogenic growth peptide (OGP). This peptide has demonstrated an
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38 ability to upregulate the differentiation of hMSCs and to promote the
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40 mineralization of the matrix. Different studies demonstrated that its
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42 capabilities are dependent to its concentration however it is
43
44 independent to the fact that the peptide is present in a soluble form
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46 of tethered on the surface of a material. (13,24,41) The OGP sequence
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48 has also been used in combination with the RGD sequence in order to
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50 differentiate pre-osteoblasts (MC3T3) into osteoblasts on polymer
51
52 substrates (polyethylene oxide). (13) It is believed that the RGD
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54 sequence binds to the integrins while the OGP sequence (no receptor
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1
2 identify up to date) causes differentiation into bone cells.(42) The
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4 combination of these two peptides might enables cells to adhere and
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6 differentiate on the same surface. Furthermore, we recently
7
8 demonstrated that the expression of OPN and RUNX2 is increased in
9
10 cells cultivated on PET surfaces tethered with RGD and OGP after two
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12 weeks, without the addition of differentiation media.(32)
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16 The level of organization of the ECM ranges from the nano- to the
17
18 micro-scale. The present work aimed to mimic the micro-organization.
19
20 Pattern shapes (squares, rectangles, hexagons) have been inspired from
21
22 previous studies that reported that elongated and angular shapes
23
24 preferentially promote the differentiation toward osteoblastic
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26 cells.(14,43,44) We have investigated similar sizes of the different
27
28 shapes of patterns with a combination of RGD and a growth factor
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30 mimicking peptide (either BMP-2 or OGP) compared to a random
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32 distribution. However, it is now demonstrated that biochemical cues
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35 distribution is not homogeneous within the ECM (45) and cells might be
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37 sensitive to different size of pattern on a surface. Therefore,
38
39 different sizes of the square patterns were also investigated.
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44 As shown in the results sections, hMSCs sense and respond to the various
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46 size of dual peptide micropatterns. Indeed, the smallest size of
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48 squares (25x25 μm^2) significantly enhanced RUNX2 expression after
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50 two weeks of cell culture, whereas the largest sizes of patterns have
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52 a similar response compared to randomly grafted peptide samples. These
53
54 results deliver indications that the hMSCs differentiation process can
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1
2 be triggered through both biochemical and geometric cues. Therefore,
3
4 the micro organization of the peptides as specific patterns appears to
5
6 be critical during the hMSCs differentiation.
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9 The use of micro-sized geometric patterns to control the stemness
10
11 character or induce stem cell differentiation is a rather recent topic.
12
13 Accordingly, few studies demonstrated the link between microscale
14
15 distribution and MSCs differentiation into specialized phenotypes.
16
17 McBeath *et al.* have used fibronectin islands onto polydimethylsiloxane
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19 of different sizes (1024, 2025, and 10 000 μm^2) to culture MSCs for 1
20
21 week in mixed osteogenic/adipogenic media.(43) The results of this
22
23 study show that MSCs cultured on the largest micro-islands mainly
24
25 differentiate into osteoblasts, whereas those on smaller micro-islands
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27 exhibits adipocytes characteristics. In our study, the most advanced
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29 differentiation process is on the smallest pattern (625 μm^2) as
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31 compared to the largest pattern (10 000 μm^2) and the random grafting.
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35 That said, McBeath *et al.* made their investigation on a single pattern
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37 element studying a single cell while the present study rather focused
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39 on arrays of biofunctionalized patterns. Therefore, the cell
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41 environment and the material on which the cells are cultured are
42
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44 different and can lead to different results. The material can have a
45
46 great impact depending on the material hydrophilicity, charge,
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48 mechanical properties.
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53 Another study investigated the fate of MSCs on RGD patterns of
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55 different geometries (circles, squares, triangles and stars) of 900
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2 μm^2 on a poly(ethylene glycol) hydrogel.(14) In this study, the optimal
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4 osteogenesis was observed on the star shape after one week of culture.
5
6 Whereas scientists still does not fully understand why specific
7
8 geometrical cues are able to induce the differentiation toward the
9
10 osteoblastic lineage, some tried to defined a general signaling
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12 pathway.(14,15,43) In the current study, the use of different shapes
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14 of grafted peptides appears to impact on the differentiation of stem
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16 cells compared to the randomly tethered surfaces. As the expression of
17
18 ColI- α 1 was significantly higher in the case of the cells cultured on
19
20 rectangle-patterned surfaces with both RGD and BMP-2, this means these
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22 cells are committed to the osteoblastic lineage.(46) In addition, the
23
24 cells in contact with the RGD/OGP peptide couple exhibit a stronger
25
26 engagement while cultured on surfaces presenting hexagonal and
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28 rectangular features. In a previous study of our group,(32) the
29
30 possible synergetic effect between RGD and OGP was investigated and it
31
32 was demonstrated that the OGP sequence is more efficient to promote an
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34 osteoblastic differentiation in presence of the RGD sequence while
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36 grafted randomly on the surface of PET. In the present study, we
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38 demonstrate that this synergetic effect between RGD and OGP peptide is
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40 further enhanced while the peptides are tethered following a specific
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42 shape (hexagonal or rectangular).
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50 Although our study provides clear evidence that hMSCs can sense
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52 geometrical cues in their environment, it appears that the size of
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54 these patterns is also an important factor to consider
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56 the differentiation process. Bilem *et al.* showed that the geometrical
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1 peptide arrangement was important to guide hMSCs differentiation using
2 smaller pattern than the ones used in the present study. (18) However,
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4
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6
7 the size of the investigated patterns in Bilem et al. work was between
8
9 10 to 200 times smaller than the patterns investigated in the present
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11 study. With the present data, cells cultured on the smallest patterns
12
13 - independently from the peptide couple investigated - exhibit strong
14
15 ~~RUNX~~RUNX-2 expression and a higher expression of ColI- α 1 and OCN in the
16
17 case of RGD/BMP-2 peptide couple. Therefore, the pattern size appears
18
19 as the most important factor to consider when designing peptide
20
21 patterns on a flat surface for hMSCs differentiation. However, since
22
23 the random grafting of peptide, which can be considered as the smallest
24
25 size of pattern we can create, did not induce further differentiation
26
27 of the cells into the osteoblastic lineage, it can be hypothesized that
28
29 cells are responsive to geometrical features until a minimum feature
30
31 size. In addition, among the three investigated markers, RUNX2 was
32
33 clearly the first to be expressed. As RUNX-2 is the first of the
34
35 three markers to be expressed during osteogenic differentiation, (46),
36
37 it is likely that the differentiation of the cells cultured on the
38
39 patterned surfaces with basal media is at an early stage, but
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41 nevertheless engaged into the process. Of note, some of the data
42
43 presented herein sometimes showed differentiation through the ColI- α 1
44
45 gene expression, without clear signs of differentiation coming from
46
47 the RUNX2 marker. However, these results were repeatedly measured. As
48
49 of now, the reason for such a behavior remains unclear.
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1
2 By combining the results of the present investigation and literature,
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4 we can draft the composite picture of an ideal biomaterial to induce
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6 a fast differentiation toward the osteoblast lineage. Indeed, according
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8 to our data, hMSCs differentiation into osteoblasts is promoted by
9
10 using a combination of RGD and BMP-2 peptide arranged on a flat
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12 surface, (18,23) tethered on the surface using sharp motifs, (47)
13
14 preferentially elongated such as rectangles, and using a pattern size
15
16 equal or smaller than $625 \mu\text{m}^2$.
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22 This ideal material would be the best one to differentiate hMSCs toward
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24 the osteoblastic lineage as our results show a higher expression of
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26 RUNX2 (4 times higher) and an expression of OCN that is twice
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28 higher (compared to the control) within 2 weeks of cell culture without
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30 using osteogenic media.
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34 Further investigations are required to fully understand these
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36 observations. However, it is likely that the smallest patterns lead to
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38 much more crosstalk between RGD and BMP-2 or OGP, therefore regulating
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40 the signalization pathways. Indeed, it is well established that RGD
41
42 peptides affect the colocalization of integrin and ligand receptors
43
44 which in turn, leads to cell commitment and differentiation. (17)
45
46
47

48 ~~45~~. Conclusion

49
50
51 2D model materials were engineered to investigate the impact of the
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53 geometry and size micro-scale distribution of RGD peptides combined
54
55 with an osteogenic inducer peptide (BMP-2 or OGP). We have recently
56

1
2 demonstrated that homogeneously co-conjugated RGD/BMP-2 or RGD/OGP
3
4 peptides onto PET surfaces significantly enhanced hMSCs osteogenesis
5
6 as compared to the solely homogeneous grafting of BMP-2 or OGP
7
8 peptides. In the present study, we demonstrated that these same
9
10 combinations of peptides can further induce stem cell differentiation
11
12 when appropriately organized on the surface. The patterning must be
13
14 relatively small (area less than $625 \mu\text{m}^2$) and sharp in terms of their
15
16 shapes (such as rectangles). Among all the concentrations that
17
18 were assessed, a 50/50 combination of RGD and BMP-2 appears to be the
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20 best mixture to promote osteogenic differentiation. Taken together,
21
22 these results suggest that the combination of chemical and geometric
23
24 cues is able to direct stem cell fate without the need of
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26 differentiation media. This surface modification strategy provides a
27
28 versatile platform for surface structuration and its optimization for
29
30 various biomaterials applications.
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53 54 **Author Contributions**

1
2 The manuscript was written through contributions of all authors. All
3
4 authors have given approval to the final version of the manuscript.

5
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25 26 27 **ABBREVIATIONS**

28
29
30 MSCs, mesenchymal stem cells; ECM, extracellular matrix; BMP-2: bone
31
32 morphogenic protein-2; PET: polyethylene terephthalate; EDC:
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34 dimethylaminopropyl-3-ethylcarbodiimideethylcarbodiimide
35
36 hydrochloride; NHS: N-hydroxysuccinimide; TBO: toluidine blue-O:
37
38 hMSCs: human mesenchymal stem cells; OCN: osteocalcin; ColI- α 1:
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40 collagen I α 1

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FIGURE CAPTIONS

Figure 1. Scheme of the different shapes and sizes of patterns

Figure 2. Scheme of preparation of the pattern surfaces

Figure 3. Profilometry images of resist micropatterned surfaces showing

three different pattern geometries (hexagons, rectangles, squares).

Figure 4. Fluorescence images of the different patterned surfaces with RGD-TAMRA (labelled in red) and OGP-FITC (labelled in green). Scale

bar: 100 μm .

Figure 5. Fluorescent intensity profile of the squared geometry with RGD-TAMRA (labelled in red) and OGP-FITC (labelled in green). Scale

bar: 100 μm .

Figure 6. Gene expression dynamics after 2 weeks of RUNX~~unx~~-2, (A: RGD+BMP; D: RGD+OGP), ColI- α 1 (B: RGD+BMP; E: RGD+OGP) and osteocalcin (OCN) (C: RGD+BMP; F: RGD+OGP) on different size of patterns (n=5).

Figure 7. Gene expression dynamics after 2 weeks of RUNX~~unx~~-2, (A: RGD+BMP; D: RGD+OGP), ColI- α 1 (B: RGD+BMP; E: RGD+OGP) and osteocalcin (OCN) (C: RGD+BMP; F: RGD+OGP) on different shapes of patterns (n=5).

TABLES

Table 1. Nucleotide sequences of primers used for quantitative RT-qPCR detection

Gene	Primer Sequence
RUNX2	5'-AAGTGCGGTGCAAACCTTTCT-3' (Forward)
	5'-TCTCGGTGGCTGGTAGTGA-3' (Reverse)
Alkaline Phosphatase	5'-ATGCCCTGGAGCTTCAGAAG-3' (Forward)
	5'-TGGTGGAGCTGACCCTTGAG-3' (Reverse)
Osteocalcin	5'-GACTGTGACGAGTTGGCTGA-3' (Forward)
	5'-CTGGAGAGGAGCAGAACTGG-3' (Reverse)
Collagen I α1	5'-ACATGTTTCAGCTTTGTGGACC-3' (Forward)
	5'-TGATTGGTGGGATGTCTTCGT-3' (reverse)
PPIA	5'-CGGGTCCTGGCATCTTGT-3' (Reverse)
	5'-CAGTCTTGGCAGTGCAGATGA-3' (Reverse)
RPC53	5'-ACCCTGGCTGACCTGACAGA-3' (Forward)
	5'-AGGAGTTGCACCCTTCCAGA-3' (Reverse)

Table 2. Expected and measured size of the pattern features for the five different geometries.

	Expected size (μm)			Measured size (μm)		
	Length	Width	Gap	Length	Width	Gap
Hexagons	88	76.2	19	81 ± 1	73.6 ± 0.5	18.7 ± 0.5
Squares 100x100	100		17	$100,2 \pm 0,8$		17.0 ± 0.7
Squares 50x50	50		15.5	49 ± 1		16.3 ± 0.7

Squares 25x25	25	9	25.2 ± 0.1	8.8 ± 0.1		
Rectangles	50	25	12.5	49.8 ± 0.6	24.8 ± 0.5	12.7 ± 0.5

For Peer

FIGURES

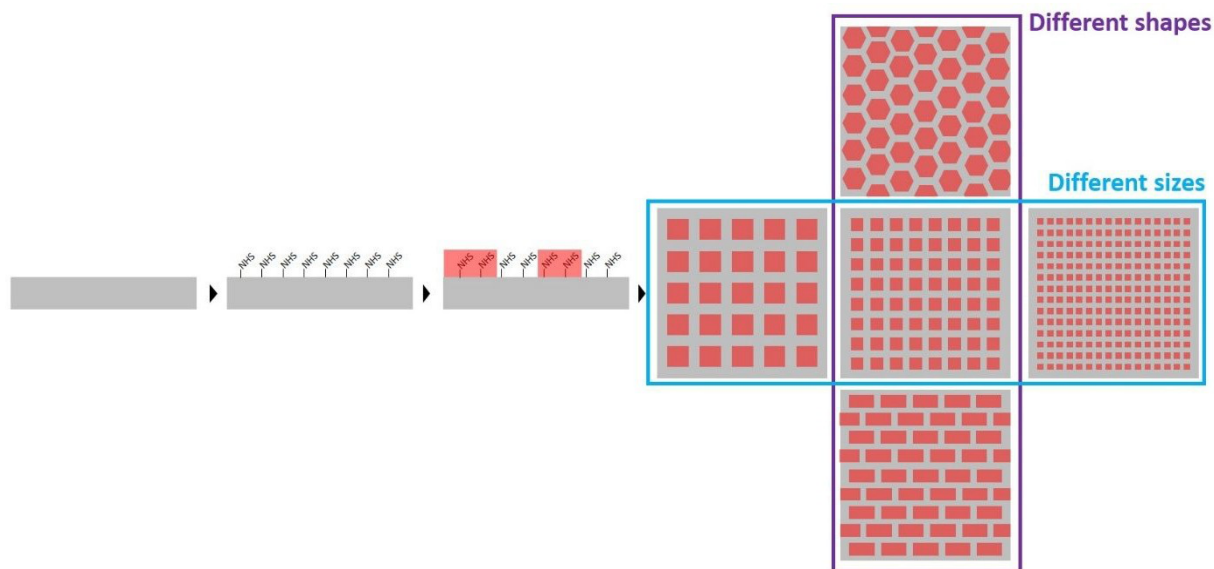


Figure 1. Scheme of the different shapes and sizes of patterns



Figure 2. Scheme of preparation of the pattern surfaces

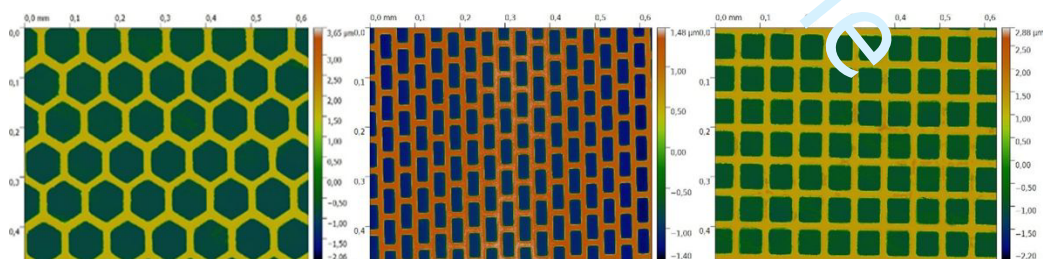


Figure 3. Profilometry images of resist micropatterned surfaces showing three different pattern geometries (hexagons, rectangles, squares).

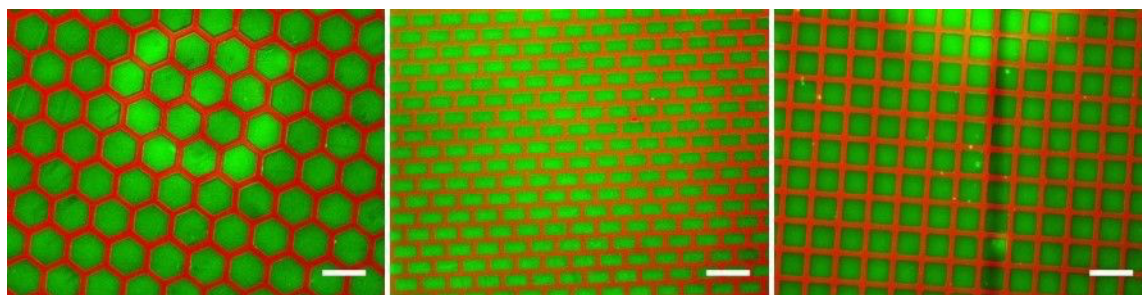


Figure 4. Fluorescence images of the different patterned surfaces with RGD-TAMRA (labelled in red) and OGP-FITC (labelled in green). Scale bar: 100 μm .

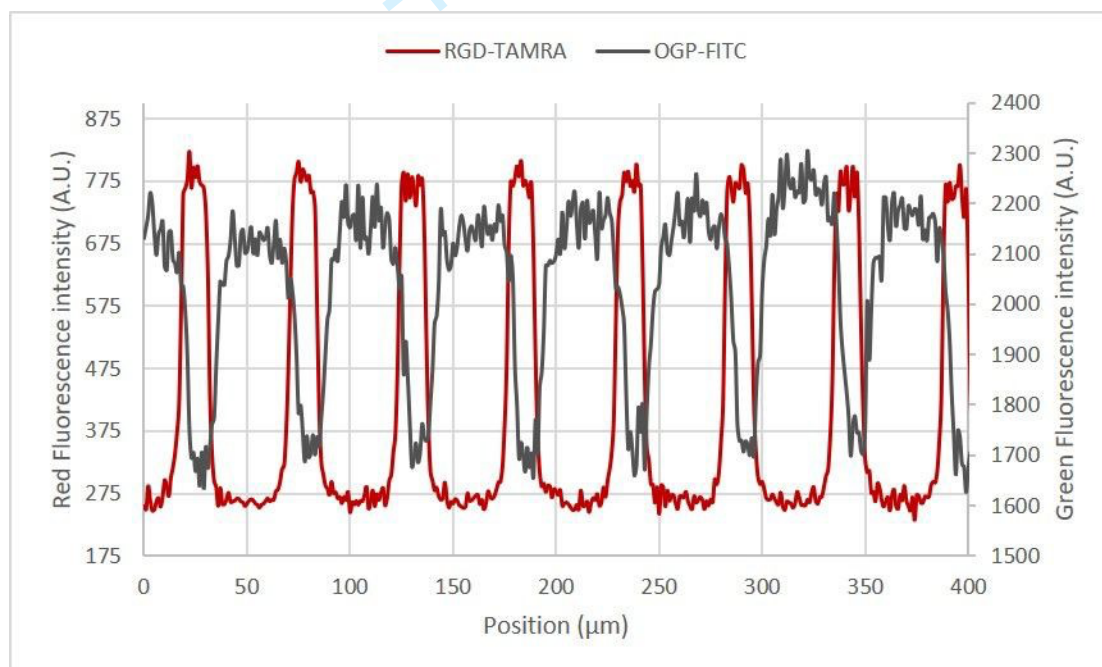


Figure 5. Fluorescent intensity profile of the squared geometry with RGD-TAMRA (labelled in red) and OGP-FITC (labelled in green). Scale bar: 100 μm .

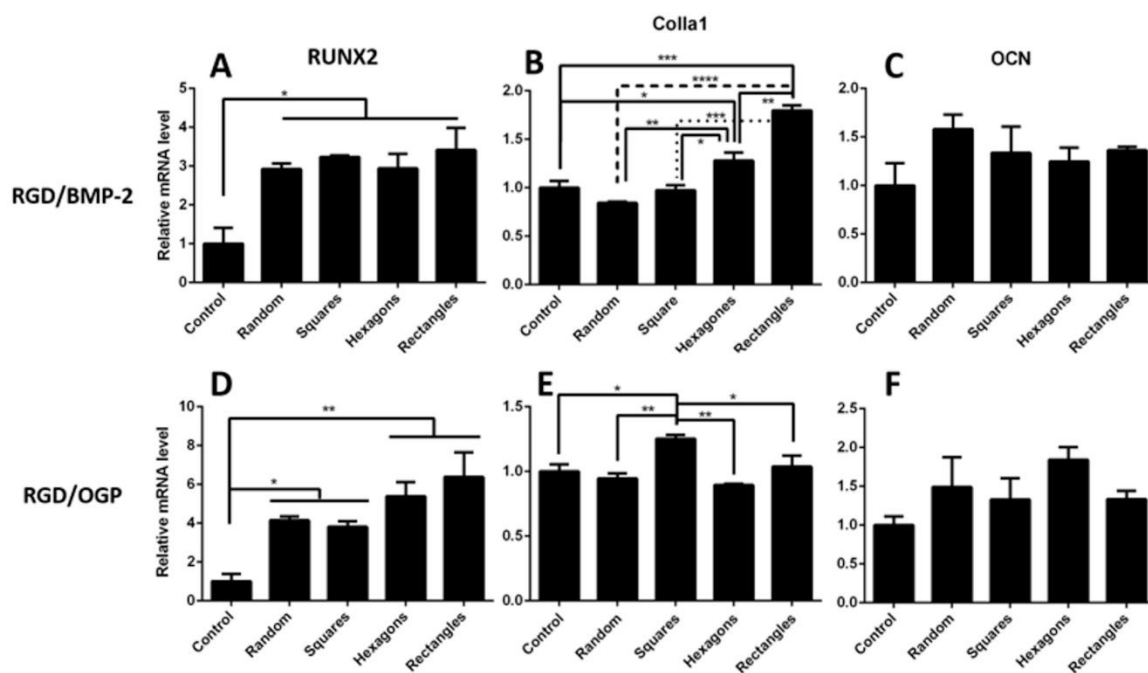
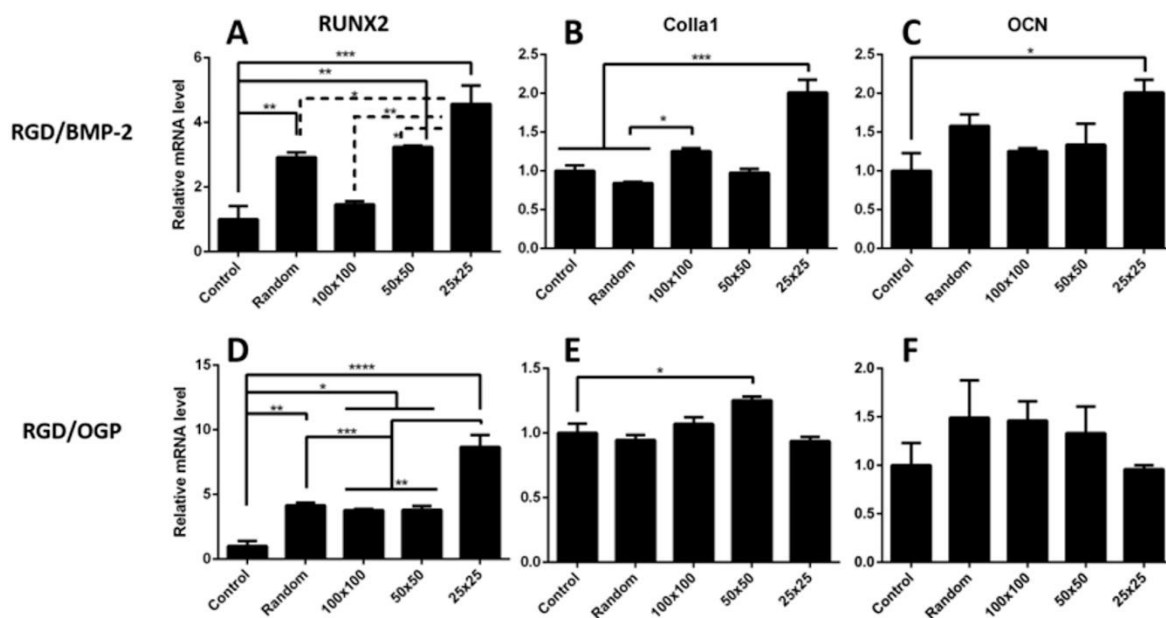


Figure 6. Gene expression dynamics after 2 weeks of ~~Runx~~-RUNX2, (A: RGD+BMP; D: RGD+OGP), ColI- α 1 (B: RGD+BMP; E: RGD+OGP) and osteocalcin (OCN) (C: RGD+BMP; F: RGD+OGP) on different size of patterns (n=5).



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Figure 7. Gene expression dynamics after 2 weeks of ~~Runx~~RUNX2, (A: RGD+BMP; D: RGD+OGP), ColI- α 1 (B: RGD+BMP; E: RGD+OGP) and osteocalcin (OCN) (C: RGD+BMP; F: RGD+OGP) on different shapes of patterns (n=5).

For Peer

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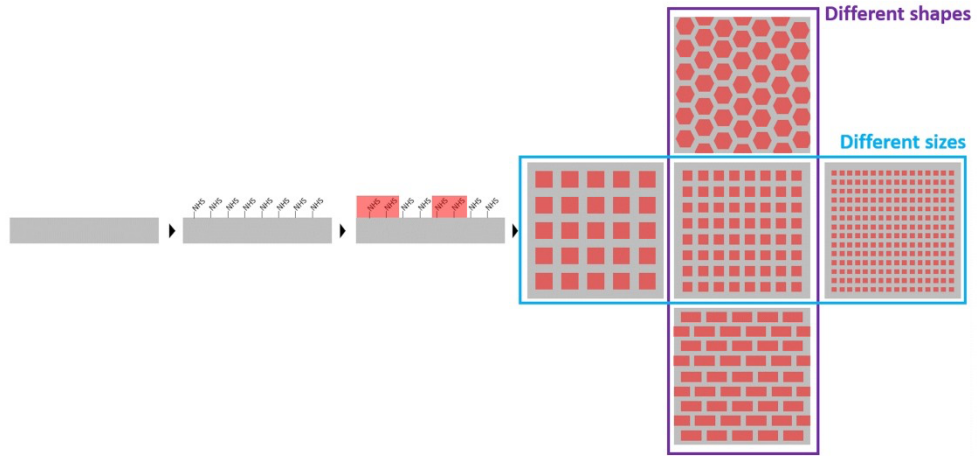


Figure 1. Scheme of the different shapes and sizes of patterns

294x139mm (300 x 300 DPI)

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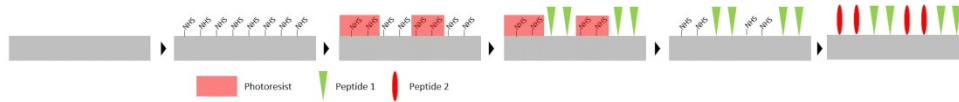


Figure 2. Scheme of preparation of the pattern surfaces
308x34mm (300 x 300 DPI)

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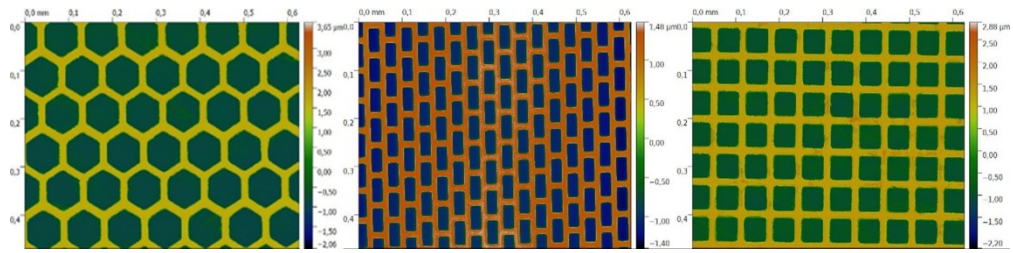


Figure 3. Profilometry images of resist micropatterned surfaces showing three different pattern geometries (hexagons, rectangles, squares).

315x75mm (300 x 300 DPI)

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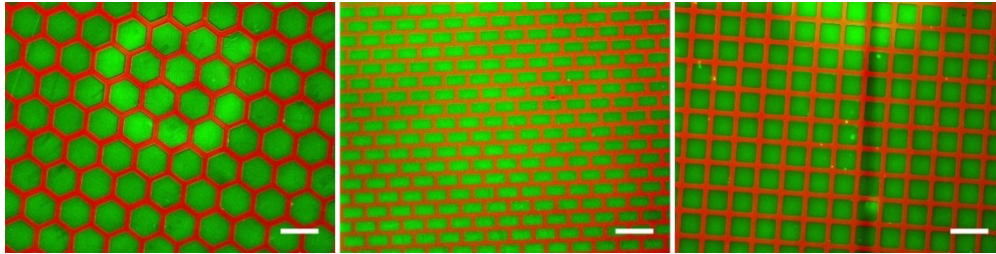


Figure 4. Fluorescence images of the different patterned surfaces with RGD-TAMRA (labelled in red) and OGP-FITC (labelled in green). Scale bar: 100 μ m.

393x99mm (300 x 300 DPI)

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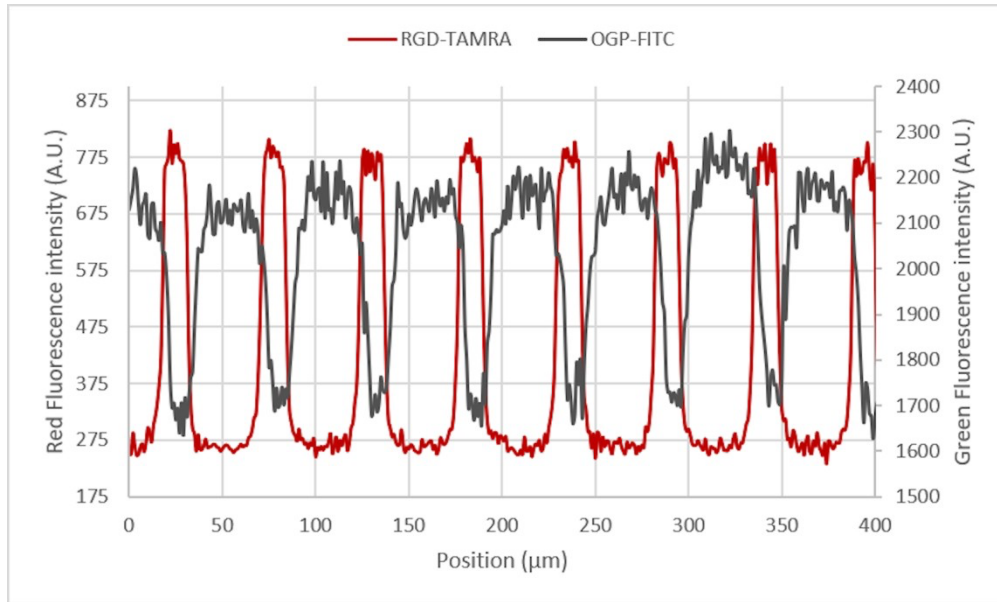


Figure 5. Fluorescent intensity profile of the squared geometry with RGD-TAMRA (labelled in red) and OGP-FITC (labelled in green). Scale bar: 100 µm.

146x87mm (300 x 300 DPI)

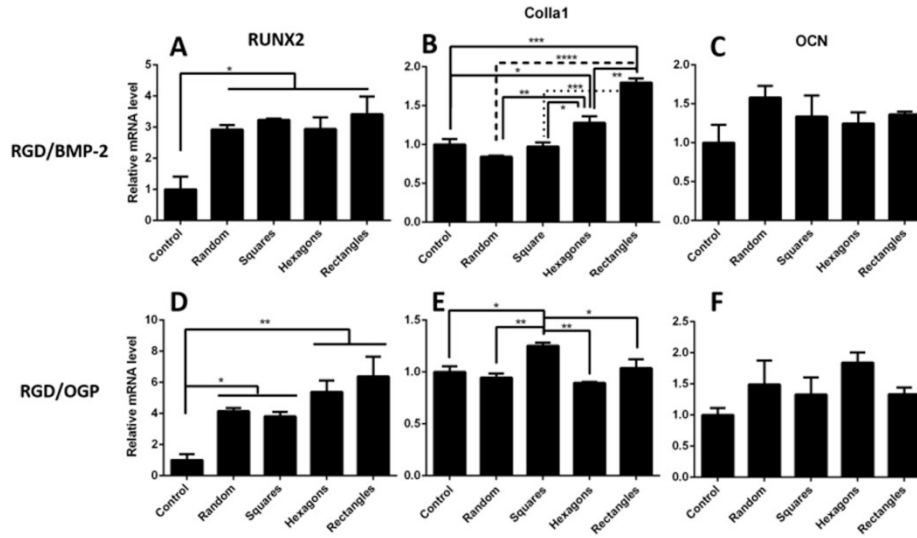


Figure 6. Gene expression dynamics after 2 weeks of RUNX2, (A: RGD+BMP; D: RGD+OGP), ColI- α 1 (B: RGD+BMP; E: RGD+OGP) and osteocalcin (OCN) (C: RGD+BMP; F: RGD+OGP) on different size of patterns (n=5).

324x181mm (300 x 300 DPI)

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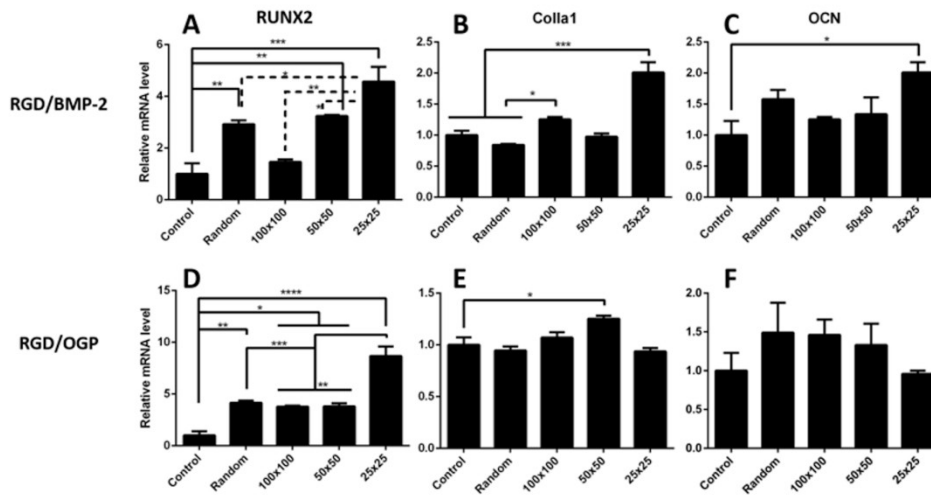


Figure 7. Gene expression dynamics after 2 weeks of RUNX2, (A: RGD+BMP; D: RGD+OGP), ColI- α 1 (B: RGD+BMP; E: RGD+OGP) and osteocalcin (OCN) (C: RGD+BMP; F: RGD+OGP) on different shapes of patterns (n=5).

323x174mm (300 x 300 DPI)

