RGD-functionalized supported lipid bilayers modulate pre-osteoblast adherence and promote osteogenic differentiation

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Abstract

Biomaterial integration into bone requires optimal surface conditions to promote osteoprogenitor behavior, which is affected by integrin-binding via arginine-glycine-aspartate (RGD). RGD-functionalized supported lipid bilayers (SLBs) might be interesting as biomaterial coating in bone regeneration, because they allow integration of proteins, for example, growth factors, cytokines, and/or antibacterial agents. Since it is unknown whether and how they affect osteoprogenitor adhesion and differentiation, the aim was to investigate adhesion, focal adhesion formation, morphology, proliferation, and osteogenic potential of pre-osteoblasts cultured on RGD-functionalized SLBs compared to unfunctionalized SLBs and poly-L-lysine (PLL). After 17 hr, pre-osteoblast density on SLBs without or with RGD was similar, but lower than on PLL. Cell surface area, elongation, and number and size of phospho-paxillin clusters were also similar. Cells on SLBs without or with RGD were smaller, more elongated, and had less and smaller phospho-paxillin clusters than on PLL. OPN expression was increased on SLBs with RGD compared to PLL. Moreover, after 1 week, COL1a1 expression was increased on SLBs with RGD compared to PLL. In conclusion, pre-osteoblast adhesion and enhanced differentiation were realized for the first time on RGD-functionalized SLBs, pointing to a new horizon in the management of bone regeneration using biomaterials. Together with SLBs nonfouling nature and the possibility of adjusting SLB fluidity and peptide content make SLBs highly promising as substrate to develop innovative biomimetic coatings for biomaterials in bone regeneration.

KEYWORDS

adherence, osteoblast, osteogenic differentiation, RGD-functionalization, supported lipid bilayer
1 INTRODUCTION

Biomaterials are used to replace bone in pathological conditions such as tooth loss, osteoarthritis, or large bone defects as a result of trauma or tumor removal (Farré-Guasch et al., 2013). In the case of tooth loss or osteoarthritis, the tooth or joint is replaced by a prosthesis, which has to integrate into the native bone. When large bone defects are concerned, resorbable grafts are inserted which in time are replaced by native bone. The integration of both implants and bone grafts starts with cell recruitment, adhesion, proliferation, and differentiation (Farré-Guasch et al., 2013; Gittens, Olivares-Navarrete, Schwartz, & Boyan, 2014; Shah, Thomson, & Palmquist, 2019). Implant integration requires that osteoprogenitors and mesenchymal stem cells are recruited, attach to the implant, proliferate, and differentiate into bone forming osteoblasts (Gittens et al., 2014; Shah et al., 2019). To allow bone graft integration, osteoclast precursors that differentiate and fuse to become bone graft resorbing osteoclasts are also needed (Farré-Guasch et al., 2013). The most important challenge in bone tissue engineering is the development of biomaterials that promote adhesion, proliferation, and differentiation of osteoprogenitors and osteoclast precursors, while repelling adhesion of bacteria, that may cause infection, and cells that produce a membranous structure between the biomaterial and the bone leading to implant failure (Gittens et al., 2014; Shah et al., 2019). For improved bone regeneration and seamless biomaterial integration into the bone, innovative biomimetic coatings for biomaterials are still needed.

Adhesion, proliferation, and differentiation of osteoprogenitors are affected by integrin binding and focal adhesion formation. Cells adhere to extracellular matrix (ECM) primarily by the binding of integrin receptors to proteins within the ECM (Sun, Guo, & Fässler, 2016). Integrin binding induces the formation of adhesion complexes where integrins cluster together, and where scaffolding and signaling proteins are recruited and attach to the actin cytoskeleton (Geiger, Spatz, & Bershadsky, 2009; Marie, Hay, & Saidak, 2014; Sun et al., 2016). Focal adhesions strengthen osteogenic cell attachment to the ECM and induce cell spreading and morphology changes by remodeling the actin cytoskeleton (Porté-Durrieu et al., 2004; Takai, Landesberg, Katz, Hung, & Guo, 2006). The signaling resulting from focal adhesion formation regulates the activity of transcription factors that direct cell growth, proliferation, survival, and differentiation toward osteoblasts (Marie et al., 2014; Sun et al., 2016; Takai et al., 2006).

Several approaches have been employed to improve the adherence and differentiation of cells on biomaterials. Improved osseointegration is observed when using implants with a rough surface compared to a smooth surface (Gittens et al., 2014; Lim et al., 2007). Surface chemistry also influences cellular adhesion (Keselowsky, Collard, & García, 2004). Cell adhesion and differentiation on biomaterials can also be improved by anchoring small peptides to the biomaterial surface. One of these peptides is arginine-glycine-aspartate (RGD), a ligand for integrins found in ECM components such as fibronectin, vitronectin, osteopontin, and bone sialoprotein (Ruoslhti & Pierschbacher, 1987). RGD has been immobilized to different substrates, including liquid crystals (Wu et al., 2017), titanium alloys (Cheng et al., 2016; Oya et al., 2009; Porté-Durrieu et al., 2004), and amine functional self-assembled monolayers (Lee et al., 2007). RGD has been shown to either have no effect on osteoprogenitor adhesion (Oya et al., 2009; Wu et al., 2017) or to positively affect osteoprogenitor adhesion (Cheng et al., 2016; Lee et al., 2007; Porté-Durrieu et al., 2004) and differentiation (Lee et al., 2007; Oya et al., 2009; Wu et al., 2017). This indicates that the functionalization of biomaterial surfaces may be improved by immobilizing RGD for optimal adhesion of osteoprogenitors.

Osteoprogenitor adhesion to RGD-functionalized biomaterials is not yet optimal possibly due to the immobile ligand presentation preventing the cells from rearranging the ECM to optimize cell–ECM interaction as occurs in vivo (Koçer & Jonkheijm, 2017). Ligand immobilization likely inhibits integrin clustering (Glazier & Salaita, 2017) and thereby decreases cell adhesion strength and signaling resulting in differentiation (Marie et al., 2014). Supported lipid bilayers (SLBs) provide a platform for functionalizing biomaterials with mobile ligands, including RGD (Glazier & Salaita, 2017; Koçer & Jonkheijm, 2017; van Weerd, Karperien, & Jonkheijm, 2015). SLBs are made of phospholipids and comparable to natural cell membranes. They are nonfouling in nature (van Weerd et al., 2015). SLBs are extensively applied into modern clinical use owing to their biophysical and chemical versatility (Ashley et al., 2011; Glazier & Salaita, 2017; Soler et al., 2018). They are applied with micro- and nano-array format, which has opened new avenues to create biochip strategies, for example, sensing strategy for diagnostics, carrier role for vaccines, theranostics, and labeling capability for imaging (Ashley et al., 2011), immunoassays(Soler et al., 2018), and tissue engineering approaches for multiple cellular processes (Glazier & Salaita, 2017).

SLBs can be functionalized with peptides derived from natural proteins, for example, ECM proteins, growth factors, cytokines, antibacterial agents, which influence cellular function. One of the intrinsic properties of SLBs is that they are fluid, that is, the phospholipids laterally diffuse through the layers (Glazier & Salaita, 2017; van Weerd et al., 2015). Since the ligands are anchored to the phospholipids, they diffuse through the lipid layers as well, facilitating the clustering of integrins and their ligands (Glazier & Salaita, 2017). The fluidity of SLBs and thereby the mobility of the ligands can be adjusted by changing the fatty acid composition and ligand density (Glazier & Salaita, 2017; Koçer & Jonkheijm, 2017; van Weerd et al., 2015). In vitro studies with mesenchymal stem cells (MSCs) (Koçer & Jonkheijm, 2017) and C2C12 myoblasts (Bennett et al., 2018) on RGD-functionalized SLBs with variable fluidity have shown contradictory results. Increased numbers of MSCs are attached to more fluid SLBs consisting of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) than to more solid SLBs consisting of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), with cells on DOPC exhibiting a larger cell area than on DPPC (Koçer & Jonkheijm, 2017). Osteogenic differentiation is enhanced in MSCs cultured on more fluid SLBs (Koçer & Jonkheijm, 2017). On the other hand, C2C12 myoblasts show a larger cell area and higher expression of myogenic markers on the more solid DPPC compared to the more fluid DOPC (Bennett et al., 2018). These studies indicate that the degree of ligand mobility modulates progenitor adhesion as well as osteogenic and myogenic differentiation.
Whether ligand mobility also modulates osteoprogenitor adhesion and differentiation is unknown.

ECM stiffness is a critical factor determining lineage commitment of MSCs (Engler, Sen, Sweeney, & Discher, 2006). Culture of MSCs on a stiff substrate (elastic modulus [E] of 25-40 kPa) results in osteogenic differentiation, whereas culture on more compliant substrates results in myogenic ([E] = 8-17 kPa) or neurogenic ([E] = 0.1-1 kPa) differentiation (Engler et al., 2006). The elastic moduli of the lipid bilayers used in the study by Koçer and Jonkheijm (Koçer & Jonkheijm, 2017) were determined by Picas et al. as 19.3 and 28.1 MPa (Picas, Rico, & Scheuring, 2012), which is much higher than the estimated elastic modulus of osteoid (27 ± 10 kPa) (Engler et al., 2006). Importantly, viscosity, regardless of matrix stiffness, also influences cell responses (Bennett et al., 2018; Charrier, Pogoda, Wells, & Janney, 2018). The mobile ligand presentation on SLBs presents the cells with a viscous component (Bennett et al., 2018). The combination of viscosity and stiffness (i.e., viscoelasticity) changes the cell response (Bennett et al., 2018). The viscoelasticity of SLBs might resemble the natural environment of osteoprogenitors (osteoid), indicating that SLBs may be a suitable substrate to stimulate osteogenic differentiation of osteoprogenitors.

Application of RGD-functionalized supported lipid bilayers as coating for biomaterials requires that osteoprogenitors, like MSCs, adhere, proliferate, and differentiate on these substrates. Therefore, the aim of this study was to investigate whether differences exist in adhesion, focal adhesion formation, morphology, proliferation, and osteogenic potential of pre-osteoblasts cultured on RGD-functionalized SLBs compared to unfunctionalized SLBs and serum-attracting poly-1-lysine (PLL), which is a commonly used pre-osteoblast culture substrate (Bakker et al., 2016; Takai et al., 2006).

This study realized for the first time pre-osteoblast adhesion and enhanced differentiation on RGD-functionalized SLBs, which could point to a new horizon in the management of bone regeneration using biomaterials. These results, together with the possibility to adjust SLB fluidity and to incorporate additional proteins that can optimize cellular function, for example, growth factors, cytokines, and/or antibacterial agents, as well as SLBs nonfouling nature make SLBs highly promising as substrate to develop innovative biomimetic coatings for biomaterials in bone regeneration. To the best of our knowledge, this is a novel approach to enhance osteoblast differentiation on biomaterials for improved bone regeneration and seamless biomaterial integration into the bone.

2 MATERIALS AND METHODS

2.1 SLB formation

SLBs were formed by vesicle fusion on a glass support as described (Koçer & Jonkheijm, 2017). Briefly, large unilamellar vesicles consisting of DOPC (melting transition temperature –20°C; Avanti Polar Lipids, Alabaster, AL) were formed by extrusion of a lipid suspension of multilamellar vesicles in MilliQ water through 100 nm membranes (Whatman Nucleopore Track-Etched polycarbonate membrane filter; Whatman, Zwijndrecht, The Netherlands). Vesicle formation was verified using dynamic light scattering (DLS; typical size 102 ± 34 nm with polydispersity index of 0.52; Microtrac Inc., Montgomeryville, PA). Vesicle suspension was sterilized by filtering through 0.2 μm membranes (Nalgene Syringe Filter; Thermo Scientific, Waltham, MA). Glass bottom wells of 96-well plates (Sensoplate, F-bottom; Greiner Bio-One, Amsterdam, The Netherlands) were incubated with 1 M NaOH for 1 hr to make the surface hydrophilic (Figure 1). After rinsing with MilliQ water, large unilamellar vesicles (0.2 mg/mL in 0.5x PBS; 100 μL/well) were put onto the glass surface and incubated for 1 hr. During incubation, the vesicles adsorbed to the glass, ruptured, and fused to form SLBs (Figure 1). After incubation, SLBs were first rinsed with PBS to remove excess vesicles, followed by rinsing with serum-free α-Modified Eagle’s Medium (α-MEM; Gibco, Paisly, UK) containing 300 μg/mL penicillin (Sigma-Aldrich, St. Louis, MO) and 250 μg/mL streptomycin (Sigma-Aldrich).

2.2 Scratch assay and fluorescent recovery after photobleaching

Homogeneous SLB formation and fluidity were confirmed by confocal microscopy. To this end, Texas Red conjugated 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (TR-DHPE, Molecular Probes, Thermo Fisher Scientific, Eugene, OR) lipid was introduced in large unilamellar vesicles at 0.2 mol %. SLBs of these vesicles were visualized using confocal microscopy (Nikon A1 confocal microscope, Nikon Instruments Europe B.V., Tokyo, Japan) to confirm homogeneous SLB formation. Scratch assays were performed by scratching the SLBs with a pipet tip and visualizing the recovery using confocal microscopy. Images were taken every 2 min. Fluorescent recovery after photobleaching was performed as described before (Koçer & Jonkheijm, 2017). Briefly, a 10 μm spot was bleached and recovery was visualized using confocal microscopy. The mobile fraction and diffusion coefficient were derived from the FRAP data using ImageJ (National Institutes of Health, Bethesda, MD) and FRAPAnalyser (University of Luxembourg, Luxembourg).

2.3 RGD functionalization

To allow cell attachment and growth, SLBs were functionalized by inserting cholesterol-conjugated RGD-peptides (chol-RGD) (Figure 1). These peptides consisting of the amino acids KGSGRGDSG were synthesized, purified, and conjugated to cholesterol using established methods available in our group (Figure S1: Mass spectrum of chol-RGD). Different concentrations of chol-RGD in PBS (0.2, 0.5, and 1.0 μM) were added to the SLBs and incubated for at least 2 hr.

2.4 Cell culture

MC3T3-E1 pre-osteoblasts were maintained in α-MEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco, Paisly, UK), 300 μg/mL penicillin (Sigma-Aldrich), 250 μg/mL streptomycin (Sigma-
Aldrich), and 1.25 μg/mL fungizone (Gibco, Paisly, UK) (Bakker et al., 2016; van Hove, Nolte, Semeins, & Klein-Nulend, 2013). At 80% confluency, the cells were harvested using 0.25% trypsin and 0.1% EDTA in PBS (Bakker et al., 2016). For experiments, cells of passage 24–34 were used. Cells were seeded at 2 × 10³ cells/cm² on different substrates and cultured for 17 hr or 1 week in α-MEM with 10% FBS, 300 μg/mL penicillin, and 250 μg/mL streptomycin. Cells were cultured for 17 hr on different substrates, since we have shown previously stable cell spreading on RGD-functionalized SLBs within this time period (Koçer & Jonkheijm, 2017). Osteogenic gene expression was measured after 1 week (Bastidas-Coral et al., 2019; van Esterik, Zandieh-Doulabi, Kleverlaan, & Klein-Nulend, 2016).

2.5 | Immunocytochemistry

To assess cell morphology and focal adhesion formation, cells were cultured for 17 hr on different substrates. Then cells were fixated using 4% paraformaldehyde in PBS and permeabilized using 0.2% Triton X-100 (Serva Electrophoresis GmbH, Heidelberg, Germany) in PBS. Nonspecific binding of antibodies was prevented by blocking with 5% normal goat serum (NGS; Life Technologies, ThermoScientific, Carlsbad, CA) and phosphorylated paxillin (phospho-Tyr31, rabbit polyclonal antibody, Invitrogen 44-720G, dilution 1:100; Fisher Scientific, Carlsbad, CA) in 5% NGS for 1 hr at room temperature. After washing five times for 10 min, cells were incubated for 1 h at room temperature with Alexa Fluor 488 goat-anti-rat (Invitrogen A11006; dilution 1:500; Fisher Scientific) and Alexa Fluor 555 goat-anti-rabbit (Invitrogen A21428, dilution 1:500; Fisher Scientific) in 5% NGS. After washing three times for 10 min with PBS, 4’,6-diamidino-2’-phenylindole dihydrochloride (DAPI) was added for 30 min at room temperature to stain the nuclei. Cells were mounted using Vectashield (Vector Laboratories Inc, Burlingame, CA).

2.6 | Confocal microscopy

Samples were imaged using a Nikon A1+ confocal laser scanning microscope (Nikon Instruments Europe B.V.). To obtain an overview of one well with cells, 36 z-stack images (slice thickness 3.0 μm) obtained with a 20x objective (numerical aperture 0.8) were stitched together in a 6 × 6 configuration. To visualize single cells, a 60x objective was used to obtain z-stack images with a slice thickness of 0.175 μm. From every well, z-stacks of 10–30 cells were taken and analyzed as described below.

2.7 | Image analysis

To investigate the cell density, a maximal z-projection was made from the stitched z-stacks using ImageJ (National Institutes of Health). An
area measuring 1000 × 1000 pixels (1253 × 1253 μm) from the center of this image was selected, and the number of cells in this image was counted using a cell counter plugin for ImageJ (De Vos, 2019). The cell density per cm² was calculated from the cell number acquired in the selected area.

For two-dimensional (2D) morphology measures, maximal z-projections were made from single cell z-stack images. Morphological parameters were measured from the resulting 2D-images using CellProfiler software (Broad Institute of Harvard and MIT, Cambridge, MA). To quantify the shape of the cell surface, elongation (eccentricity, equals 1 for a straight line and 0 for a perfect circle), circularity (form factor, 2π × area/perimeter; Shah et al., 2019, equals 1 for a perfect circle), and extent (approaches 1 for compact cells without protrusions) were quantified. Cell volume was measured from single cell z-stacks using Medical Imaging Interaction Toolkit software (MITK; German Cancer Research Center, Heidelberg, Germany).

To analyze focal adhesions, summated z-projections of the lower 15 slices of single cell images were made using ImageJ. From the resulting 2D images, integrin α5 staining intensity and phosphopaxillin cluster number and size were measured using ImageJ as described (Horzum, Ozdil, & Pesen-Okvur, 2014).

### 2.8 | Gene expression analysis

After 17 hr or 1 week of culture, cells were lysed using TRIreagent (Invitrogen; Fisher Scientific). Total RNA was extracted using RNAqueous-micro kit (Invitrogen) according to the manufacturers’ protocol and measured using nanodrop 2000 (Thermo scientific). Complementary DNA (cDNA) was synthesized using 100 ng RNA for cells cultured for 17 hr, and 200 ng RNA for cells cultured for 1 week in 20 μL reaction mixture using Superscript VILO MasterMix (Invitrogen). For each target gene 5 μL of 10× diluted cDNA was amplified in duplicate using Fast SYBR™ Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA) on a StepOne Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific). Target proliferation marker genes Ki67 and CCND1 and osteogenic marker genes RUNX2, OPN, COL1α1, and ALP were analyzed. Gene expression levels were calculated relative to the housekeeping gene HPRT1 using the delta Ct method. Primer sequences are listed in Table 1.

#### TABLE 1 | Primer sequences used for real-time PCR

| Target gene | Primer sequence | Annealing temperature (°C) |
|-------------|----------------|---------------------------|
| PXNα | Forward: 5’-CAGTCCCGACGGAGTCA-3’<br>Reverse: 5’-CCTGGGCACTGAATTGAAATC-3’ | 60 |
| PXNβ | Forward: 5’-ACCAGGGAGAGATGAGCAGT-3’<br>Reverse: 5’-AGGCCCTCATGACTGAAATCT-3’ | 60 |
| ITGA5 | Forward: 5’-GGAGGGAGGGAGCTGACT-3’<br>Reverse: 5’-TAGACAGACACACCTGAG-3’ | 60 |
| Ki67 | Forward: 5’-CCCTCAAGCAAGCTGAGAA-3’<br>Reverse: 5’-AGAGCCTATATAGGAGCG-3’ | 60 |
| CCND1 | Forward: 5’-TCAAGTGTGACCCGGACTG-3’<br>Reverse: 5’-GACCTCAAGGCGTCTCATT-3’ | 60 |
| RUNX2 | Forward: 5’-ATTACAGATCCCCAGGCAGGC-3’<br>Reverse: 5’-TCTGATCTGAGTAGGGG-3’ | 60 |
| OPN | Forward: 5’-CCCGGTGAAAGTGACTGATT-3’<br>Reverse: 5’-TTCCGTACTCGAACGGGAAT-3’ | 60 |
| COL1α1 | Forward: 5’-AACGTCGATACATCGCAGCG-3’<br>Reverse: 5’-TTCCGTACCTGAGGCCAAAT-3’ | 60 |
| ALP | Forward: 5’-GGACCAACCTGACTGACCCT-3’<br>Reverse: 5’-CCCTCTCCACCCAGCAAGAA-3’ | 60 |
| HPRT1 | Forward: 5’-CCTAAGATGAGCGCAAGTGAA-3’<br>Reverse: 5’-CCACGAGCTAGAACCACCCTGTA-3’ | 60 |

**Abbreviations:** ALP, alkaline phosphatase; COL1α1, collagen type I α1 chain; HPRT1, hypoxanthine phosphoribosyltransferase 1; PXNα, paxillin transcript variant α; PXNβ, paxillin transcript variant β; ITGA5, integrin α5; CCND1, cyclin D1; RUNX2, runt-related transcription factor 2; OPN, osteopontin.
as an indication that at least one substrate differed from at least one of the other substrates. When a significant interaction effect was observed, differences were further investigated by repeating the mixed model ANOVA with PLL excluded, which always resulted in a nonsignificant interaction effect, indicating that the distribution of the measurement in cells on PLL was different from that of the measurement on other substrates. To investigate whether the percentage of cells was different between substrates in a certain category, one-way ANOVA with Bonferroni post hoc tests (or Kruskall–Wallis tests with pairwise comparisons if parametric methods were not appropriate) was performed for every category. Mean ± SEM was calculated and significant differences tested using Kruskall–Wallis test with pairwise comparisons, since data were not normally distributed.

3 | RESULTS

3.1 | Functionalized glass slides with SLB

Homogeneous coatings of SLBs conjugated with Texas Red-DHPE (1,2-dihexadecanoyl-sn-glycero-3-phosphoethanol-amine) were formed on glass surfaces as shown by confocal microscopy (Figure 2a). The fluidity of the SLBs was demonstrated by showing that a scratch in the layer gradually disappeared resulting in full SLB recovery (Figure 2b). Fluorescent recovery after photobleaching revealed recovery of fluorescence over time, also demonstrating the fluidity of the SLBs (Figure 2c). In an earlier study we determined the diffusion coefficient (0.91 ± 0.21 μm²) and the mobile fraction (90.3 ± 3.5%) (Koçer & Jonkheijm, 2017).

3.2 | Cell density

To investigate the adherence of MC3T3-E1 pre-osteoblasts on SLBs with and without RGD, the number of cells per cm² was quantified (Figure 3). Cells did adhere to SLBs with and without RGD. Cell density on SLBs with RGD (1353 ± 255 cells/cm², mean ± SEM of 3 RGD-concentrations used) and on SLBs without RGD (1621 ± 266 cells/cm²) was 30–45% lower than on PLL-coated glass (2299 ± 426 cells/cm²; Figure 3; p < .001). There were no significant differences in cell density between SLBs with and without RGD.

3.3 | Cell morphology

To investigate the effect of RGD-functionalized supported lipid bilayers on cellular morphology, parameters for cell size (cell surface area, cell volume) and shape of the surface area (elongation, circularity, and...
Table 2: Effect of SLBs without or with RGD on MC3T3-E1 pre-osteoblast morphology after 17 hr of culture

| Surface area (μm²) | Volume (μm³) | Eccentricity | Form factor | Extent |
|-------------------|-------------|--------------|-------------|--------|
| PLL               | 3114 ± 279  | 8929 ± 1421  | 0.77 ± 0.02 | 0.14 ± 0.02 | 0.36 ± 0.03 |
| SLB               | 1652 ± 283  | 6652 ± 1281  | 0.85 ± 0.04 | 0.14 ± 0.04 | 0.31 ± 0.07 |
| +0.2 μM RGD       | 1821 ± 235  | 6118 ± 664   | 0.89 ± 0.01 | 0.11 ± 0.01 | 0.28 ± 0.03 |
| +0.5 μM RGD       | 2146 ± 194a | 5963 ± 1019  | 0.91 ± 0.01 | 0.10 ± 0.01 | 0.21 ± 0.03 |
| +1.0 μM RGD       | 1666 ± 233  | 5963 ± 382   | 0.88 ± 0.01 | 0.12 ± 0.02 | 0.31 ± 0.04 |
| SLB ± RGD         | 1763 ± 227* | 6174 ± 737** | 0.87 ± 0.02** | 0.12 ± 0.02** | 0.30 ± 0.04** |

Notes: The morphological parameter values for surface area, volume, eccentricity, form factor, and extent are mean ± SEM. SLB ± RGD values are mean ± SEM of pooled data of all SLB experimental groups. Cells on SLBs without or with RGD had a smaller surface area, smaller volume, larger eccentricity, smaller form factor, and lower extent than cells on PLL. Cells on SLBs with 0.5 μM RGD exhibited a larger surface area than cells on SLBs without RGD or with 1.0 μM RGD. N > 300 cells. Significantly different from PLL, *p < .05, **p < .001. "Significant effect of RGD, p < .05. Abbreviations: PLL, pol-L-lysine; SLB, supported lipid bilayer.

3.4 | Focal adhesion formation

Focal adhesion formation in MC3T3-E1 pre-osteoblasts on the different substrates was investigated by measuring the number and size of phosphorylated paxillin clusters, as a measure for the number and size of focal adhesions formed, and the intensity of integrin α5 staining as a measure for the number of integrins present in the cell (Figure 5). Cells on SLBs with or without RGD showed less and smaller clusters of phospho-paxillin than cells on PLL-coated glass (Figure 5b,c; p < .001). Cells cultured on SLBs with 0.5 μM RGD showed more phospho-paxillin clusters than cells cultured on SLBs with 0.2 μM RGD or 1.0 μM RGD (Figure 5c; p < .05). No differences in integrin α5 staining intensity between substrates were observed (Figure 5d). There were no differences in the levels of paxillin mRNA (both transcript variant α and β) between substrates (Figure 5e,f). ITGA5 mRNA content did not differ between substrates (Figure 5g).

3.5 | Proliferation

To investigate whether RGD-functionalized SLBs influence proliferation of MC3T3-E1 pre-osteoblasts we investigated gene expression of Ki67 and Cyclin D1 (CCND1) (Figure 6). After 17 hr, the expression levels of both Ki67 and CCND1 in cells cultured on SLBs without RGD or on SLBs with 0.5 or 1.0 μM RGD were similar to those in cells cultured on PLL-coated glass (Figure 6a). There was a trend toward upregulation of Ki67 and CCND1 expression on SLBs with 0.2 μM RGD compared to SLBs without RGD (not significant). After 1 week, Ki67 and CCND1 expression did not differ between substrates, and were lower than after 17 hr of culture (Figure 6b).

3.6 | Osteogenic differentiation state after 17 hr of culture

To investigate the initial osteogenic gene expression response to RGD-functionalized SLBs, RUNX2, osteopontin (OPN), collagen type I (COL1a1), and alkaline phosphatase (ALP) gene expression was analyzed (Figure 6a). After 17 hr of culture, gene expression levels of OPN were 2.5-fold higher in cells cultured on SLBs with 0.2 μM RGD compared to those cultured on PLL-coated glass (Figure 6a; p < .05).
trend toward 1.5-fold higher mRNA expression levels of the early osteogenic marker **RUNX2** in cells cultured on SLBs with 0.2 μM RGD compared to those cultured on PLL-coated glass (Figure 6a; n.s.). There were no differences regarding the expression of the late osteogenic marker **COL1α1** between the different substrates (Figure 6a). **ALP** expression, also a late osteogenic marker, was too low to be determined (Figure 6a).
3.7 | Osteogenic differentiation state after 1 week of culture

To further elucidate the effect of RGD-functionalized SLBs on osteogenic differentiation of pre-osteoblasts, gene expression of RUNX2, OPN, COL1a1, and ALP was also analyzed after 1 week of culture (Figure 6b). There was a 1.4-fold higher expression of COL1a1 in cells cultured on SLBs with 0.5 μM RGD than on PLL-coated glass (Figure 6b; *p < .02). In cells cultured on SLBs without RGD and with 0.2 μM and 1.0 μM RGD, the mean expression of COL1a1 mRNA was 1.4-fold higher than in cells cultured on PLL (Figure 6b; n.s.). Compared to 17 hr, gene expression levels of OPN, COL1a1, and ALP were higher after 1 week, while RUNX2 expression was lower (Figure 6). This shows that after 1 week the expression of osteogenic genes in cells cultured on RGD-functionalized SLBs was comparable to the expression in cells cultured on PLL, if not higher.

4 | DISCUSSION

This study aimed to investigate whether differences exist in adhesion, morphology, focal adhesion formation, proliferation, and osteogenic potential of pre-osteoblasts on RGD-functionalized SLBs compared to unfunctionalized SLBs and PLL-coated glass substrate. We showed for the first time that pre-osteoblasts did adhere to SLBs with and without RGD, albeit 30–45% less than to PLL-coated glass; (b) cells cultured on SLBs with and without RGD were ~35% smaller and 10% more elongated with more protrusions than cells cultured on PLL-coated glass; (c) cells cultured on PLL-coated glass showed 70–85% more and ~50% larger phospho-paxillin clusters than cells cultured on SLBs with and without RGD; (d) osteopontin mRNA expression levels were 2.5-fold higher in cells cultured for 17 hr on SLBs with 0.2 μM RGD than in those cultured on PLL; (e) after 1 week of culture there was a trend towards increased COL1a1 expression in cells cultured on SLBs compared to PLL. These results suggest that pre-osteoblasts cultured on SLBs with RGD were more osteogenic than cells on PLL-coated glass, despite their smaller size and lower phospho-paxillin content, indicating that application of RGD-functionalized SLBs with variable fluidities to study the mechanisms underlying cell fate and function in relation to physical and chemical substrate properties is promising, as well as clinical application of RGD-functionalized SLBs as coating on biomaterials for enhanced bone regeneration and osteointegration.

4.1 | Reduced adhesion of pre-osteoblasts on RGD-functionalized SLBs compared to PLL-coated glass

After 17 hr of culture, cell density on SLBs without or with RGD was lower than on PLL-coated glass, which might be explained by the larger variety of ligands for cell attachment on PLL-coated substrates than on SLBs. PLL is a positively charged molecule that interacts with negatively charged sites on adhesion complexes at cell surfaces (Zimmerman et al., 2009). The positive charge of PLL molecules likely allows serum proteins to adsorb to the glass, which provides a multitude of ligands for integrin receptors. SLBs are nonfouling, that is, serum proteins do not or barely adsorb, preventing cells from attaching to the surface (van Weerd et al., 2015). Therefore, negligible numbers of ligands for integrins are presented on SLBs, unless they are functionalized with integrin-binding peptides, such as the RGD peptide used in this study (van Weerd et al., 2015). RGD-functionalized SLBs present only one type of ligand for integrins, while serum proteins adsorbed to PLL probably contain additional peptide sequences that serve as ligands for other integrins, such as sequences in collagen to which a different class of integrins attaches. Therefore, the variety of ligands for integrins was likely larger on PLL-coated substrates than on RGD-functionalized SLBs, providing cells with more opportunities to adhere.

In this study, cell adhesion on SLBs with and without RGD was similar. This is unexpected and might reveal a lack of interaction between Chol-RGD and SLBs. However, this is highly unlikely, since quartz crystal microbalance with dissipation monitoring (QCM-D) revealed successful interaction between Chol-RGD and SLB (data not shown). Therefore, it is unlikely that the similar cell densities on SLBs...
FIGURE 5  Effect of SLBs without or with RGD on focal adhesion formation in MC3T3-E1 pre-osteoblasts after 17 hr of culture. (a) Typical examples of cells on PLL, SLBs without RGD, and SLBs with increasing concentrations of RGD. Cells were stained for integrin α5 (green), phospho-paxillin (yellow), and nuclei (blue). Bar: 50 μm. (b) Number of phospho-paxillin clusters per cell. Cells on PLL had more phospho-paxillin clusters than cells on SLBs with or without RGD (p < .001). The number of phospho-paxillin clusters was higher in cells cultured on SLBs with 0.5 μM RGD then in cells cultured on SLBs with 0.2 μM or 1.0 μM RGD (p < .05). n = 4 separate experiments, 30–40 cells/substrate/experiment. (c) Mean phospho-paxillin cluster area per cell. The area of phospho-paxillin clusters was larger in cells cultured on PLL than in cells cultured on SLBs with or without RGD (p < .001). The mean area of phospho-paxillin clusters in cells cultured on SLBs without RGD was larger than in cells cultured on SLBs +0.2 μM RGD (p < .05). n = 4 separate experiments, 30–40 cells/substrate/experiment. (d) Mean integrin α5 staining intensity per cell. n = 4 separate experiments, 30–40 cells/substrate/experiment (in total 150–200 cells/experiment). (e) Relative mRNA levels of paxillin transcript variant α. n = 5 separate experiments. (f) Relative mRNA levels of paxillin transcript variant β. n = 5 separate experiments. (g) Relative integrin α5 gene expression. n = 5 separate experiments. **p < .001, *p < .05. PLL, poly-L-lysine coated glass; SLBs, supported lipid bilayers.
FIGURE 6  Effect of SLBs without or with RGD on proliferation-related and osteogenic gene expression in MC3T3-E1 pre-osteoblasts after 17 hr and 1 week of culture. (a) Gene expression in MC3T3-E1 pre-osteoblasts after 17 hr of culture. Relative mRNA expression of Ki67 and CCND1, as well as RUNX2 was similar in cells cultured on the different substrates. OPN expression was higher in cells cultured on SLBs with 0.2 μM RGD than on PLL (p < .05). COL1α1 expression was not different between cells cultured on the different substrates. ALP expression was too low to be determined. (b) Gene expression in MC3T3-E1 pre-osteoblasts after 1 week of culture. Relative mRNA expression of proliferation markers Ki67 and CCND1, as well as osteogenic markers RUNX2, OPN, and ALP were similar in cells cultured on the different substrates. COL1α1 expression was increased in cells cultured on SLBs compared to PLL. n = 5. *p < .05; **p < .02; CCND1, Cyclin D1; OPN, osteopontin; SLBs, supported lipid bilayers; PLL, poly-L-lysine coated glass; COL1α1, collagen type I α1 chain; ALP, alkaline phosphatase; n.d., not detectable
with and without RGD can be explained by a lack of interaction between Chol-RGD and SLB. It also seems unlikely that the similar cell densities on SLBs with and without RGD result from a charged surface on SLBs. Mazia, Schatten, and Sale (1975) have performed electrokinetic measurements showing that the zeta potential of DOPC-SLBs approaches zero with increasing salt concentrations up to 10 mM KCl at pH 7.4. In our study, we used α-MEM with 5.3 mM KCl and 117 mM NaCl at pH 7.4. With this high ionic strength, the zeta potential is expected to be nearly zero. Therefore, it seems unlikely that the effect of SLBs without RGD on pre-osteoblasts is the result of surface charge.

4.2 | Ligand mobility and density on RGD-functionalized SLBs affect pre-osteoblast morphology

Osteoblasts cultured on SLBs with and without RGD were generally smaller, more elongated, and showed more protrusions than cells cultured on PLL-coated glass. Our findings that the cell surface area was smaller and more elongated, are similar to those observed for MSCs on RGD-functionalized SLBs (Koçer & Jonkheijm, 2017). C2C12 myoblasts cultured on RGD-functionalized SLBs are also smaller than myoblasts cultured on glass (Bennett et al., 2018). This indicates that cells spread less on RGD-functionalized SLBs than they do on serum-coated glass.

The smaller cells on RGD-functionalized SLBs in comparison to serum-coated glass may be explained by possible variation in types of integrin ligands present on these substrates. It is also possible that cell morphology is affected by the mobile ligand presentation on the viscoelastic SLBs. Cells sense their environment by pulling on their attachments and sensing the resistance of the matrix to this pulling (Discher, Janmey, & Wang, 2005). Since RGDs in the SLBs can diffuse through the bilayer, pulling of the cells on their attachments may displace these attachments towards the center of the cell, causing a smaller cell area on SLBs. Therefore, SLB fluidity affects cell morphology and behavior.

Not only variety and lateral mobility of the ligands but also variation in RGD density may determine cell spreading. Our data show that cells cultured on SLBs with 0.5 μM RGD were larger than cells cultured on SLBs with 1.0 μM RGD, suggesting that there is an optimum ligand density for cell spreading on RGD-functionalized SLBs. The density of immobilized RGD-peptides has been shown to be positively related to MC3T3-E1 cell area (Arnold et al., 2004; Arnold et al., 2008; Huang et al., 2009). However, in these studies, cells were cultured on very stiff (glass) substrates, while substrate stiffness affects the relation between ligand density and cell area (Oria et al., 2017). Since the SLBs used in the current study have a lower elastic modulus than glass (Picas et al., 2012) and present RGD-peptides in a mobile manner as consequence of the SLB fluidity (Bennett et al., 2018; Glazer & Salaita, 2017), the relationship between ligand density and cell spreading is possibly different on SLBs with mobile RGD-peptides compared to substrates with immobilized RGD-peptides. SLBs provide the opportunity to investigate in detail the relationship between pre-osteoblast spreading and substrate stiffness, ligand variety, mobility, and density. Nevertheless, this is only relevant if a relationship between pre-osteoblast spreading and osteogenic phenotype exists, but such a relationship has not been established. Interestingly, MSCs with a large surface area are more osteogenic than MSCs with a small surface area (Frith, Mills, & Cooper-White, 2012; Guo, Lu, Merkel, Sterling, & Guelcher, 2016; Koçer & Jonkheijm, 2017; Wang et al., 2013). Therefore, for studies investigating the effect of substrate stiffness and ligand presentation on pre-osteoblast morphology, it should be established whether there is a relation between pre-osteoblast morphology and the osteogenic state.

4.3 | Reduced focal adhesion formation in pre-osteoblasts cultured on RGD-functionalized SLBs compared to PLL indicating lower adhesion strength

Cells on SLBs without and with RGD showed less and smaller phospho-paxillin clusters than cells on PLL-coated glass. These differences likely resulted from differences in phosphorylation of paxillin and not from differences in protein content, since there were no differences in paxillin mRNA. The size of phospho-paxillin clusters is indicative of the strength of the adhesions and the forces applied to the adhesions, either by contraction of the actin cytoskeleton or as a result of external mechanical perturbations (Marie et al., 2014). Therefore, the decreased number and smaller phospho-paxillin clusters on SLBs compared to PLL-coated glass suggest that cells did adhere less firm to SLBs, which substantiates the reduced cell adhesion shown in the current study.

Cells on SLBs with 0.5 μM RGD showed more phospho-paxillin clusters than on SLBs with 0.2 or 1.0 μM RGD. This indicates that ligand density on the fluid SLBs modulated focal adhesion formation, as did the density of immobile ligands (Burridge, Turner, & Romer, 1992).

Phosphorylation of paxillin not only implies adhesion but also activation of focal adhesion kinase and other signaling molecules in the adhesion complex (Khatiwala, Kim, Peyton, & Putnam, 2009). These signaling molecules activate signaling pathways such as mitogen-activated protein kinase, which play a critical role in osteogenic differentiation of MSCs by stimulating RUNX2 gene expression (Khatiwala et al., 2009; Marie et al., 2014). MSCs with large focal adhesions are more osteogenic than cells with small focal adhesions (Frith et al., 2012; Guo et al., 2016; Koçer & Jonkheijm, 2017; Wang et al., 2013). MC3T3-E1 pre-osteoblasts show increased focal adhesion formation accompanied by decreased osteocalcin expression and matrix mineralization (Kong, Polte, Alsberg, & Mooney, 2005). Therefore, it is unclear whether the less abundant and smaller focal adhesions in pre-osteoblasts cultured on RGD-functionalized SLBs in comparison to PLL-coated glass indicated that cells were more or less osteogenic on SLBs than on PLL. Thus, we also investigated the effect of RGD-functionalized SLBs on osteogenic gene expression.
4.4 Effect of RGD-functionalized SLBs on osteogenic gene expression

To further assess the initial response of pre-osteoblasts to RGD-functionalized SLBs, we examined their osteogenic state by analyzing osteogenic gene expression. Cells cultured on SLBs with and without RGD for 17 hr did express the osteogenic genes RUNX2, COL1a1, and OPN. Cells on SLBs with 0.2 μM RGD showed increased OPN expression and a trend toward elevated RUNX2 expression compared to PLL.

The ECM protein osteopontin is expressed in bone, as well as in other organs, for example, kidney, heart, and inner ear (De Fusco et al., 2017; Denhardt & Guo, 1993). It is a signaling molecule that is upregulated in osteoblasts in response to mechanical loading (Young, Gerard-O’Riley, Kim, & Pavalko, 2009). Osteopontin is an essential protein in bone involved in matrix remodeling and tissue calcification (De Fusco et al., 2017). Thus, the higher OPN expression in pre-osteoblasts cultured on SLBs suggest that these cells were more osteogenic than on PLL-coated glass.

The 17 hr culture period in our study was rather short to investigate the osteogenic phenotype of pre-osteoblasts. Changes in gene expression of RUNX2, COL1a1, and ALP are usually only observed after several days of culture (Bastidas-Coral et al., 2019; van Esterik et al., 2016). MSCs cultured for 10 days on RGD-functionalized SLBs are more osteogenic, that is, they show increased ALP activity when more fluid SLBs are used compared to less fluid SLBs (Koçer & Jonkheijm, 2017), indicating that initial culturing of MSCs on more rigid RGD-functionalized SLBs guides osteogenic differentiation of MSCs. Therefore, the osteogenic state of MC3T3-E1 pre-osteoblasts after 1 week of culture on RGD-functionalized SLBs was investigated. It was observed that the cells were still well attached to the surface and nicely spread. Compared to the 17 hr time point, the cell layer was more confluent, indicating that cells had proliferated on SLBs.

Gene expression levels of RUNX2 and OPN on all substrates were lower after 1 week than after 17 hr of culture. In contrast, the expression levels of COL1a1 and ALP were higher after 1 week than after 17 hr. These results are in line with the normal osteogenic differentiation pattern (van Esterik et al., 2016), showing that pre-osteoblasts grow and differentiate well on RGD-functionalized SLBs.

Pre-osteoblasts cultured on SLBs showed increased expression of COL1a1 compared to cells cultured on PLL, suggesting that pre-osteoblasts cultured on SLBs may be more osteogenic than on PLL. The lack of effect on expression of other osteogenic genes may be the result of the possible degradation of the SLBs over time during culture. This degradation of SLBs has been shown for charged SLBs consisting of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) during culture of neuronal cells (Afanasenkau & Offenhäusser, 2012). Degradation of SLBs may allow serum proteins to adsorb to the hydrophilic glass creating an environment comparable to that on PLL-coated glass. If so, the slightly higher expression of COL1a1 in cells cultured on SLBs suggests that the initial culturing on SLBs induces signaling that stimulates COL1a1 expression even after the SLBs have started to degrade. To further elucidate the long-term effect of SLBs on cells, SLBs exhibiting higher stability are required. This can be achieved by, for example, polymerization of diacetylene-containing lipids introduced within the SLB (Morigaki et al., 2013). An important consideration while targeting SLB stability is preserving SLB fluidity, that is, its biomimicry as desired for certain biomedical applications (Deng et al., 2008; Morigaki et al., 2013).

4.5 Future perspectives for SLBs as coating for biomaterials in bone

Taken together, the results of this study are a first indication that SLBs may be promising as coating for biomaterials in bone, although SLBs have to be further developed to optimize adhesion and differentiation of osteoprogenitors. Changing fatty acid composition and thereby the lateral mobility of SLBs and attached ligands likely affects osteoprogenitor adhesion and differentiation. Osteoprogenitors adhere and differentiate better on substrates with higher rigidity (Wang et al., 2013). Preparing SLBs of lipids with a higher melting transition temperature and thereby lower lateral mobility (e.g., 1-myristol-2-palmitoyl-sn-glycero-3-phosphocholine [MPPC], melting transition temperature 35 °C) may provide an environment where cells experience slightly more resistance when pulling on their attachments, increasing focal adhesion formation and thereby adhesion strength and probably also osteogenic differentiation.

Another way to optimize SLBs for osteoprogenitor adhesion and differentiation may be SLB functionalization with more than one peptide. Immobilization of the short peptide GFOGER, the major binding locus for integrins on collagen type I, to nonfouling substrates induces adhesion of osteoblasts to a level comparable to adhesion on full collagen type I-coated substrates (Reyes & García, 2003). Furthermore, immobilization of RGD together with its synergy sequence PHSRN to polyethylene glycol hydrogels improves osteoblast adhesion compared to RGD alone (Benoit & Anseth, 2005). Osteoprogenitors also adhere with a higher affinity to RGD-peptides with a cyclic conformation than to RGD-peptides with a linear conformation (Porté-Durrieu et al., 2004). The current study used a peptide with a linear conformation and therefore adhesion can likely be improved by functionalizing SLBs with a cyclic RGD peptide. Furthermore, peptides derived from growth factors or cytokines can be incorporated into the SLBs to optimize cellular function, for example, vascular endothelial growth factor (VEGF) to stimulate vascularization.

An advantage of SLBs is their nonfouling nature. This intrinsic resistance of SLBs to the adsorption of proteins and cellular adhesion likely prevents bacteria from attaching to the surface, lowering the risk of infection when SLBs are used as a coating for biomaterials. To further reduce the infection risk, antimicrobial proteins/peptides can be incorporated into the SLBs as well (Chen & Chen, 2006). Future research will have to elucidate how changing fatty acid composition of the SLBs and functionalization with other peptides can be used to develop innovative coatings for biomaterials in bone regeneration.
CONCLUSIONS

This study realized for the first time pre-osteoblast adhesion and enhanced differentiation on RGD-functionalized SLBs, which could point to a new horizon in the management of bone regeneration using biomaterials. These results, together with the possibility to adjust SLB fluidity and to incorporate additional proteins that can optimize cellular function, for example, growth factors, cytokines and/or antibacterial agents, as well as SLBs non-fouling nature make SLBs highly promising as substrate to develop innovative biomimetic coatings for biomaterials in bone regeneration.

ACKNOWLEDGMENTS

The authors acknowledge Dr. M.L. Verheijden for help with peptide synthesis and purification. The work of J. Jin was granted by the China Scholarship Council (CSC, No. 201608530156). M. Haroon was funded by the European Commission through MOVE-AGE, an Erasmus Mundus Joint Doctorate programme (Grant number: 2014-0691).

CONFLICT OF INTEREST

All authors have no conflict of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Verstappen JFM, Jin J, Koer G, et al. RGD-functionalized supported lipid bilayers modulate pre-osteoblast adherence and promote osteogenic differentiation. *J Biomed Mater Res. 2020;108:923–937. https://doi.org/10.1002/jbm.a.36870*