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Surface guidance of stem cell behavior: Chemically tailored co-presentation of integrin-binding peptides stimulates osteogenic differentiation *in vitro* and bone formation *in vivo*

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ABSTRACT

Surface modification stands out as a versatile technique to create instructive biomaterials that are able to actively direct stem cell fate. Chemical functionalization of titanium has been used in this work to stimulate the differentiation of human mesenchymal stem cells (hMSCs) into the osteoblastic lineage, by covalently anchoring a synthetic double-branched molecule (PTF) to the metal that allows a finely controlled presentation of peptidic motifs. In detail, the effect of the RGD adhesive peptide and its synergy motif PHSRN is studied, comparing a random distribution of the two peptides with the chemically-tailored disposition within the custom made synthetic platform, which mimics the interspacing between the motifs observed in fibronectin. Contact angle measurement and XPS analysis are used to prove the efficiency of functionalization. We demonstrate that, by rationally designing ligands, stem cell response can be efficiently guided towards the osteogenic phenotype: *In vitro*, PTF-functionalized surfaces support hMSCs adhesion, with higher cell area and formation of focal contacts, expression of the integrin receptor $\alpha5\beta1$ and the osteogenic marker Runx2, and deposition a highly mineralized matrix, reaching values of mineralization comparable to fibronectin. Our strategy is also demonstrated to be efficient in promoting new bone growth *in vivo* in a rat calvarial defect. These results highlight the efficacy of chemical control over the presentation of bioactive peptides; such systems may be used to engineer bioactive surfaces with improved osseointegrative properties, or can be easily tuned to generate multi-functional coatings requiring a tailored disposition of the peptidic motifs.

Statement of significance

Organic coatings have been proposed as a solution to foster osseointegration of orthopedic implants. Among them, extracellular matrix-derived peptide motifs are an interesting biomimetic strategy to harness cell-surface interactions. Nonetheless, the combination of multiple peptide motifs in a controlled manner is essential to achieve receptor specificity and fully exploit the potentiality of synthetic peptides. Herein, we covalently graft to titanium a double branched molecule to guide stem cell fate *in vitro* and generate an osseoinductive titanium surface *in vivo*. Such synthetic ligand allows for the simultaneous presentation of two bioactive motifs, thus is ideal to test the effect of synergic sequences, such as RGD and PHSRN, and is a clear example of the versatility and feasibility of rationally designed biomolecules.

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1. Introduction

Several therapies for the treatment of injured bone tissue are currently established, yet recent findings on the natural healing processes of the organism suggest new routes for improvement. The role of stem cells in the restoration of damaged tissue has been fully recognized [1,2], and much effort is nowadays dedicated to the understanding of the underlying mechanisms [3], with a view to establishing novel clinical treatments [4,5]. Mesenchymal stem cells (MSCs) are non-haematopoietic, heterogeneous pluripotent cells that are capable of differentiating into several mesodermal and non-mesodermal cell lineages [6]. As traumatic injury occurs, these progenitor cells are mobilized from their niche and recruited to the damaged tissue, in order to contribute to the reparative process [7]. Their contribution to the natural healing response of the body has been described to be via *in situ* differentiation into cells that directly replace the damaged tissue and paracrine action (or trophic activity [8]), which controls the injury-related inflammatory response [9].

Particular attention has been given to the engineering of biomaterials that control the commitment of MSCs to specific lineages, such as neuronal [10], chondrogenic [11], cardiac [12], and osteoblastic [13]. However, harnessing MSC fate remains a major challenge. Addressing such challenge would be of great significance in the orthopaedic and maxillofacial field, where the capacity to stimulate the osteogenic differentiation of MSC on the surface of clinically-relevant materials (e.g. on titanium and its alloys) [14] would translate into higher rates of implant osteointegration and improved long-term functionality. Surface modification strategies can be used for this purpose. As a matter of fact, variations of the surface stiffness [15], chemical composition [16,17], topography [18,19], and hydrophilicity [20] have been proven to influence MSC response.

Among chemistry-based strategies of surface functionalization, the anchoring of integrin-binding ligands is a particularly interesting solution to guide osteodifferentiation [21,22]. Integrins are a family of heterodimeric transmembrane receptors, constituted by two non-covalently bound α and β subunits, that are responsible for the communication of signals from the extracellular matrix (ECM) to the nucleus, and vice versa [23]. Although, a complete view on how integrin ligands influence the response of progenitor cells has not been achieved yet, peptides derived from the ECM have been used to modulate cell fate. This is the case of the two integrin-binding motifs present in the cell attachment site of fibronectin (FN): the Arg-Gly-Asp (RGD) peptide [24], which interacts with several integrin subtypes [25], and the Pro-His-Ser-Arg-Asn (PHSRN) motif, whose synergic effect increases the affinity of RGD for the integrin receptor α5β1 [26]. This integrin receptor has been proved to be important in osteogenesis and thus its specific engagement opens promising prospects in bone regeneration [22,27–29]. Presentation of RGD and PHSRN sequences at the proper distance is crucial to preserve their synergic behavior [30,31]. The effect of these two ECM-derived motifs on cell adhesion has been studied in the literature [30,32–35], but their capacity to induce osteogenic differentiation of MSCs on metallic substrates has been only achieved by using engineered recombinant fragment of FN encompassing the whole cell attachment site of the protein [36–38]. Nonetheless, the limitations associated to the use of proteins urge the development of alternative synthetic approaches that mimic and retain their integrin-binding specificity but offer higher chemical control, safety and stability [39]. To mimic the 30–40 Å distance that separates the motifs in FN [40,41] within a synthetic ligand, several linkers have been proposed, including polyglycine chains, $(Gly)_6$ [42,43] or $(Gly)_{13}$ [35], as well as $(Ser\text{-}Gly)_5$ units [32]. In our study we use a novel design based on a double-branched molecule (Fig. 1), which uses 4

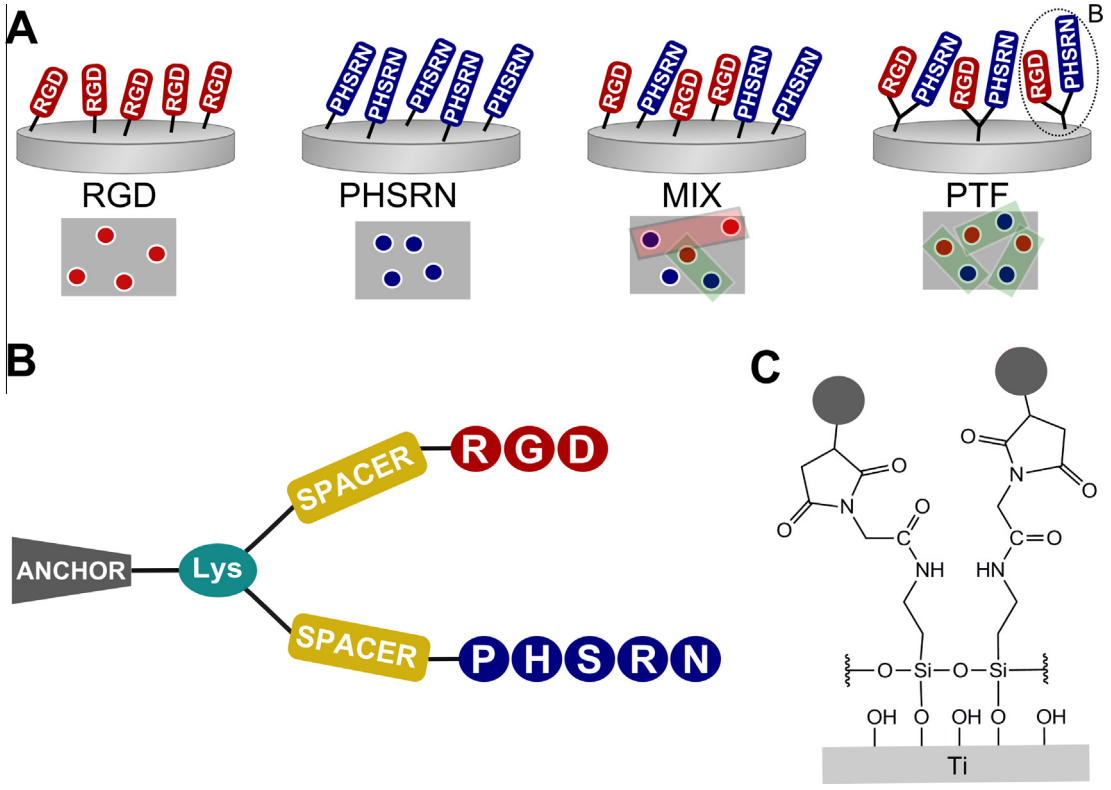

Fig. 1. Schematic representation of the functionalized surfaces (A) and of the double-branched platform (B). Silanization of the Ti substrate is used to covalently graft the ligands (grey spots) (C).