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Hydrogels functionalized with N-cadherin mimetic peptide enhance osteogenesis of hMSCs by emulating the osteogenic niche

Meiling Zhu [a, b], Sien Lin [c], Yuxin Sun [c], Qian Feng [a, b], Gang Li [c], Liming Bian [a, b, d, *]

[a] Division of Biomedical Engineering, The Chinese University of Hong Kong, Hong Kong, People's Republic of China
[b] Department of Mechanical and Automation Engineering, The Chinese University of Hong Kong, Hong Kong, People's Republic of China
[c] Department of Orthopaedics and Traumatology, The Chinese University of Hong Kong, Hong Kong, People's Republic of China
[d] Shun Hing Institute of Advanced Engineering, The Chinese University of Hong Kong, People's Republic of China

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ABSTRACT

N-cadherin is considered to be the key factor in directing cell–cell interactions during mesenchymal condensation, which is essential to osteogenesis. In this study, hyaluronic acid (HA) hydrogels are bio-functionalized with an N-cadherin mimetic peptide to mimic the pro-osteogenic niche in the endosteal space to promote the osteogenesis of human mesenchymal stem cells (hMSCs). Results show that the conjugation of the N-cadherin peptide in the HA hydrogels enhances the expression of the osteogenic marker genes in the seeded hMSCs. Furthermore, the biofunctionalized HA hydrogels promote the alkaline phosphatase activity, type I collagen deposition, and matrix mineralization by the seeded hMSCs under both *in vitro* and *in vivo* condition. We postulate that the biofunctionalized hydrogels emulates the N-cadherin-mediated homotypic cell–cell adhesion among MSCs and the "orthotypic" interaction between the osteoblasts and MSCs. These findings demonstrate that the biofunctionalized HA hydrogels provide a supportive niche microenvironment for the osteogenesis of hMSCs.

1. Introduction

Cell–cell interactions and the formation of tight mesenchymal aggregates during the mesenchymal condensation are the earliest morphogenetic events preceding the development of several musculoskeletal tissues such as cartilage and bone. Bone formation is a complex process involving osteoprogenitor cells and their progressive differentiation into mature functional osteoblasts. N-cadherin is a transmembrane protein considered to be the key factor in directing cell–cell interactions during mesenchymal condensation [1,2]. Blocking cell–cell adhesion by using specific peptides or antibodies in MSCs or osteoblastic cells leads to reduced osteoblast differentiation and bone nodule formation *in vitro* [3–5]. Furthermore, studies have shown that the expression of the dominant-negative mutant of N-cadherin gene in osteoblasts results in impaired osteogenesis, delayed bone mass development, and a shift from osteogenesis to adipogenesis in bone marrow mesenchymal stem cells (MSCs) [6]. The deletion of N-cadherin in osteoblasts reduces β-catenin abundance and decreases cell–cell adhesions between bone marrow stromal cells and calvaria bone cells, potentially leading to a reduced osteoprecursor population in the bone marrow [7]. These studies reveal the key role of N-cadherin in mediating not only interactions between differentiating MSCs but also interactions between the osteoblasts and MSCs in the bone marrow niche, which are crucial to the osteogenesis of MSCs and bone development.

HA hydrogels prove to be a bioactive material with which MSCs can interact through cell surface receptors including CD44 and CD168. The interactions between HA and its receptors regulate multiple cellular behaviors of MSCs including proliferation and osteogenic differentiation [8,9]. Our earlier work has shown that HA hydrogel conjugated with an N-cadherin mimetic peptide containing the "HAV" tripeptide sequence, which is a conserved motif in the first extracellular domain of the classic type I cadherins responsible for the homotypic binding of cadherins [10], promotes the chondrogenesis of the encapsulated hMSCs [11]. However, few previous studies have examined the effect of the N-cadherin mimetic peptide on regulating hMSCs osteogenesis. Thus, in this study, we functionalized 2D HA hydrogel substrates and 3D porous

* Corresponding author. Room 213, William M.W. Mong Engineering Building, Department of Mechanical and Automation Engineering, The Chinese University of Hong Kong, Shatin, Hong Kong, People's Republic of China.
E-mail address: lbian@mae.cuhk.edu.hk (L. Bian).

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HA hydrogel constructs with the N-cadherin mimetic peptide to emulate the cell–cell interactions during mesenchymal condensation and to mimic the osteoblast–MSC interaction in the bone marrow niche that are critical to the osteogenesis. RGD peptide is also conjugated to the HA hydrogels in all experimental groups to allow cell adhesion because the N-cadherin peptide alone does not support cell adhesion to the HA hydrogel based on our preliminary data. We hypothesize that the conjugation of the N-cadherin peptide in the HA hydrogels will enhance osteogenic differentiation of the seeded hMSCs, thereby leading to more bone matrix deposition in the hydrogels.

2. Results

2.1. The N-cadherin expression in hMSCs is dependent on the degree of osteogenesis

Human MSCs were first cultured on 2D HA hydrogel substrates that were conjugated with RGD peptide only in osteogenic media (Fig. 1A, Fig. 2B, Fig. S1). Immunofluorescence staining against N-cadherin shows that hMSCs express a copious amount of the membranous N-cadherin at the beginning of the culture (Fig. S2). The amount of N-cadherin gradually decreases with increasing osteogenic culture time and becomes undetectable by day 14 of the culture (Fig. S2). This is consistent with previous studies that have reported diminishing N-cadherin expression with the increasing maturation of the osteoblastic cells, thus indicating that N-cadherin is involved in the early stage of osteogenesis [4,5,12,13].

2.2. The conjugation of N-cadherin mimetic peptide on 2D HA hydrogel substrates enhances the osteogenesis of the seeded hMSCs

During osteogenic culture, hMSCs seeded on the 2D HA hydrogel substrates that are conjugated with both the N-cadherin mimetic and RGD peptide (Cad + RGD) form increasingly larger cell clusters (Fig. 2A). On day 12, cell clusters on the Cad + RGD hydrogels substrate are significantly larger compared to those on the control substrates (Scram + RGD, RGD) (Fig. 2AD). ALP staining shows a significantly higher percentage of stain cells on the N-cadherin peptide (Cad + RGD: 89%) than that of the control groups (Scram + RGD: 75%; RGD: 61%) (Fig. 2CE). The immunofluorescence staining reveals more intense (two-fold increase) Runx2 staining in the Cad + RGD group compared to the control groups (Scram + RGD, RGD) (Fig. 2FG). The Cad + RGD group also shows a higher level of ALP activity after 20 days and 28 days of osteogenic culture (Fig. S3). After 20 days of osteogenic culture, the 2D hydrogels that are conjugated with N-cadherin peptide (Cad + RGD) appear more opaque than the controls (Scram + RGD, RGD) (Fig. S4A); this is likely caused by mineral deposition. Von Kossa staining reveals more calcium deposition in the Cad + RGD group than the control groups, and this is consistent with the ALP activity result (Fig. S4B).

2.3. Addition of unconjugated N-cadherin mimetic peptide in media inhibits the osteogenesis of hMSCs

To test the effect of unconjugated N-cadherin peptide on hMSCs, free diffusing N-cadherin peptide is supplemented in the osteogenic media. ALP staining shows that the addition of free N-cadherin peptide significantly inhibits the osteogenesis of the hMSCs cultured on HA hydrogels conjugated with RGD peptide only (Fig. S5B). In contrast, the supplementation of free scrambled peptide has no such inhibitory effect (Fig. S5C). Consistent with previous findings, when the N-cadherin peptide is conjugated on the hydrogel substrates the seeded hMSCs exhibit a significantly higher ALP activity compared to the control (Fig. S5A). Thus, the

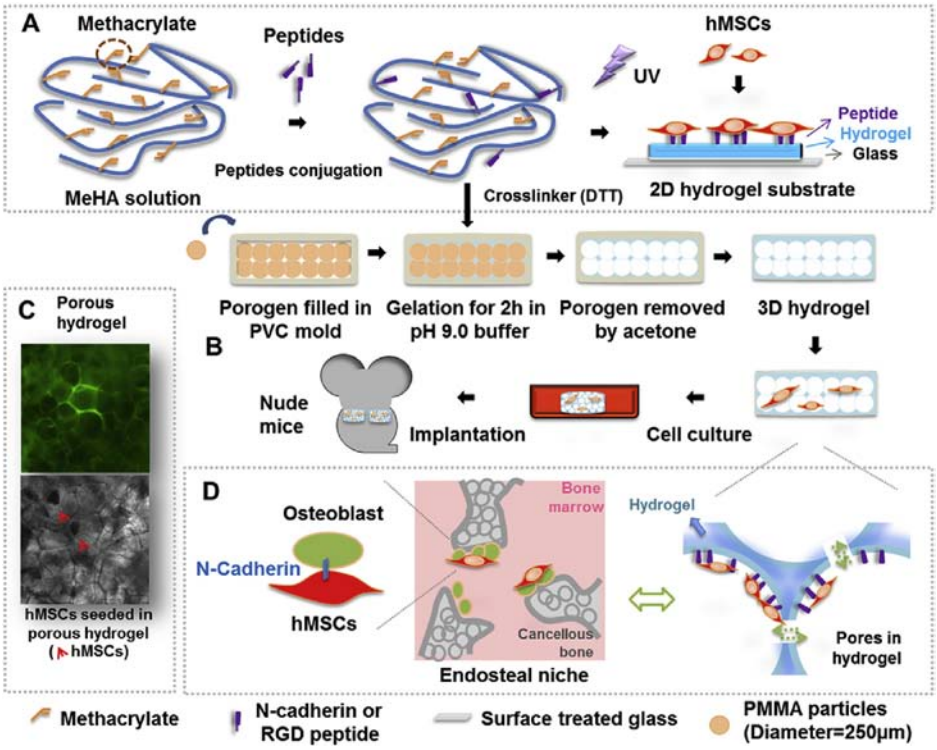

Fig. 1. Peptide conjugation and hMSCs seeding on (A) 2D HA hydrogel substrates and (B) 3D porous HA hydrogels for *in vitro* culture or subcutaneous implantation in nude mice. (C) Micrographs of the 3D porous hydrogels. (D) Comparison between the endosteal niche and the biomimetic porous hydrogels.

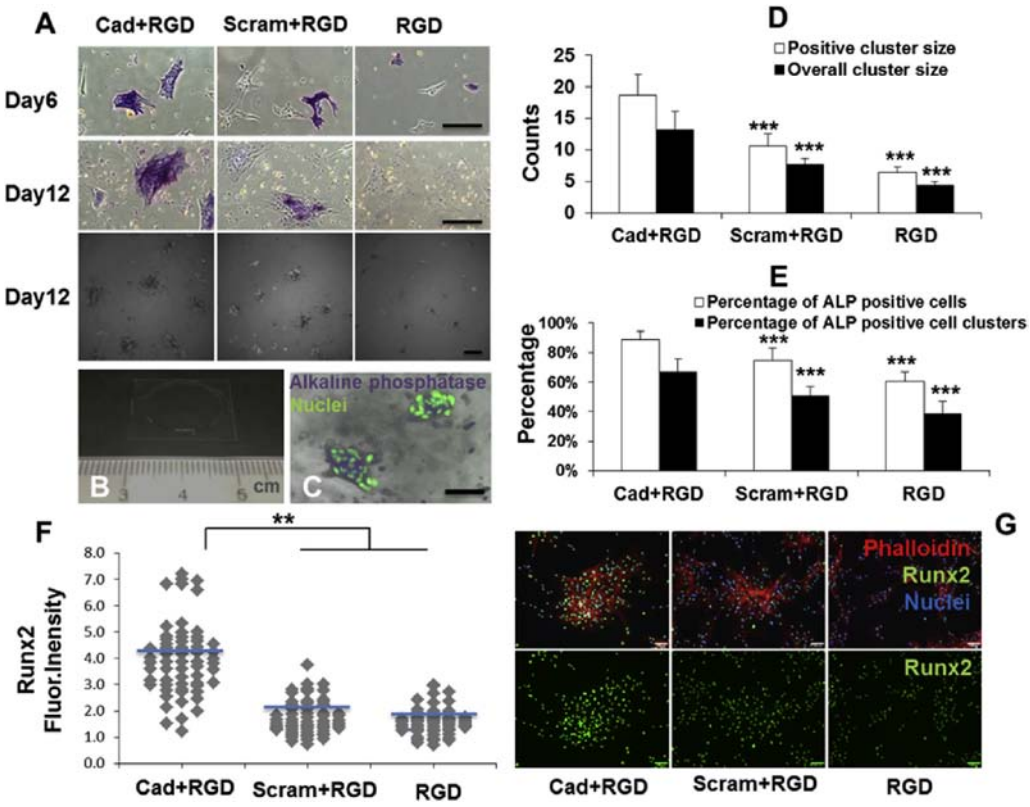

Fig. 2. Osteogenesis of the seeded hMSCs on N-cadherin mimetic peptide conjugated 2D HA hydrogel substrates. (A) Alkaline phosphatase staining of the hMSCs cultured on 2D hydrogel substrates after 6 and 12 days of osteogenic differentiation. (Scale bar = 250 μm) (B) Image of the 2D hydrogel substrate. (C) Representative micrograph of ALP and nuclei staining (pseudo color) of the hMSCs seeded on 2D hydrogel substrates. (Scale bar = 250 μm) (D) ALP positive and overall (including ALP negative clusters) cell cluster size and (E) percentage of ALP positive cells cultured on 2D peptide functionalized HA hydrogel substrates after 12 days of osteogenic culture. (F) Runx2 fluorescent intensity (intensity ratio between nuclei and cytoplasm, n = 60, horizontal lines represent the mean) and (G) Runx2 immunostaining of the hMSCs seeded on 2D hydrogel substrates after six days of osteogenic culture. (Scale bar = 100 μm) (**p value < 0.01, ***p value < 0.001, vs Cad + RGD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

free diffusing and conjugated N-cadherin peptide has a distinctively different effect on the osteogenesis of hMSCs.

2.4. Conjugation of the N-cadherin peptide in 3D porous hydrogels enhances the osteogenesis of the seeded hMSCs in vitro

Cell viability staining indicates that the conjugation of RGD peptide on the 3D porous peptide functionalized HA hydrogels is necessary for hMSC adhesion to the hydrogels. Few cells are found in the non-peptide-conjugated 3D porous hydrogels (Fig. S6A), whereas significantly more hMSCs adhere to the 3D porous hydrogels conjugated with the RGD peptide (Fig. S6BC). Compared to the control hydrogels (RGD), the porous hydrogels conjugated with the N-cadherin peptide (Cad + RGD) significantly upregulated the mRNA expression of osteogenic markers including type I collagen (148% higher), osteocalcin (106% higher), ALP (295% higher) and Runx2 (104% higher) in the seeded hMSCs after four days of osteogenic differentiation (Fig. 3A). The hydrogels conjugated with the scrambled peptide (Scram + RGD) show no such upregulated gene expression (Fig. 3A). The upregulated expression of the osteogenic markers in the Cad + RGD group persists on day 12 of the culture, albeit to a lesser extent (Fig. 3A). Furthermore, the Cad + RGD group develops a significantly higher content of calcium after 24 days of culture compared to the controls (Scram + RGD, RGD) (Fig. 3B). To be specific, when normalized by the BCA content, the Cad + RGD group has 70% more calcium compared to the Scram + RGD and the RGD control group (Fig. 3B). Similarly, the ALP activity of the Cad + RGD group is 37% higher than that of the control groups (Fig. 3CD). Von Kossa staining of the tissue sections shows that the Cad + RGD group accumulates significantly more calcification than the control groups (Scram + RGD, RGD) (Fig. 3E). Immunohistochemical staining reveals more intense staining against type I collagen and Runx2 in the porous hydrogels conjugated with N-cadherin mimetic peptide compared to the control groups (Fig. 3E).

2.5. Bio-functionalization of HA hydrogels with N-cadherin peptide enhances the osteogenesis of the seeded hMSCs in vivo

After 28 days of implantation, the hMSC-laden 3D porous hydrogels functionalized with the N-cadherin peptide (Cad + RGD) appear to attract more blood vessels than the control groups (Cad + RGD, RGD) (Fig. 4A). Blood-vessel-like structures can be observed in the paraffin sections of the implanted 3D porous hydrogels stained with the hematoxylin/eosin staining (Fig. S7). Furthermore, various analyses reveal a similar trend in the calcium content and the bone matrix synthesis in the implanted hydrogels as that observed in the *in vitro* experiments. As evidenced by the calcium quantification and Von Kossa staining, the calcium content of the Cad + RGD group is significantly higher compared to the Scram + RGD and RGD groups when normalized with sample weight, though the total calcium content was lower than that in the *in vitro* study (Fig. 4B). Both the immunohistochemical staining against type I collagen and the hematoxylin/eosin staining on the histological sections of hMSCs-laden 3D porous hydrogels show more bone matrix in the Cad + RGD group than in the Scram + RGD

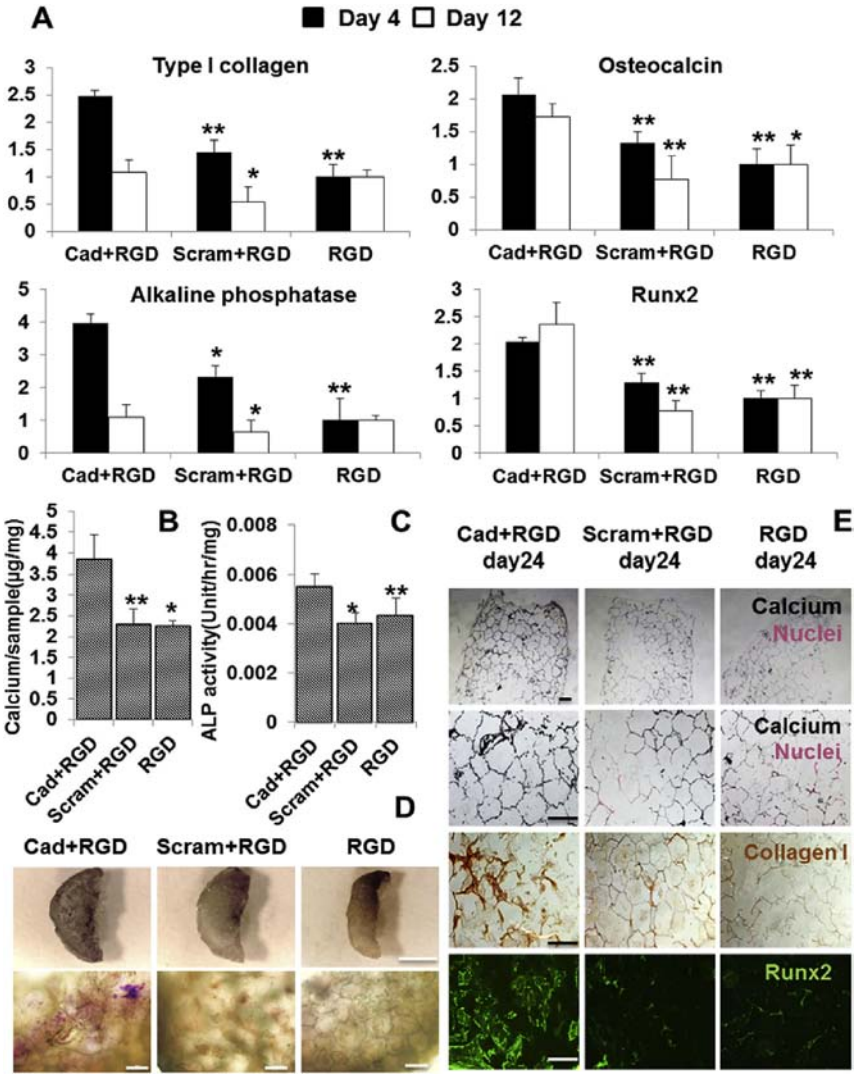

Fig. 3. Osteogenesis of the seeded hMSCs in N-cadherin peptide conjugated 3D porous hydrogels in vitro. (A) Gene expression of osteogenic markers in the hMSCs-laden 3D porous peptide functionalized HA hydrogels after four days and 12 days of osteogenic differentiation. (*p value < 0.05, **p value < 0.01 vs Cad + RGD of day 4 or day 12, n = 4) (B) Calcium content (day 12), (C) ALP activity level (day 12) (*p value < 0.05, **p value < 0.01 vs Cad + RGD, n = 4), and (D) alkaline phosphatase staining (day 24) of the hMSC-laden 3D porous peptide-functionalized HA hydrogels during osteogenic culture. (Scale bar: upper row = 2 mm; lower row = 200 μm) (E) Von Kossa staining (nuclear red as the counterstain), and immunohistochemical staining against type I collagen and Runx2 of the paraffin sections of the hMSC-laden 3D porous peptide-functionalized HA hydrogels after 24 days of osteogenic differentiation. (Scale bar = 250 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and RGD groups (Fig. 4CD).

2.6. Rat MSCs-laden porous hydrogels functionalized with N-cadherin peptide promote healing of calvarial defects in rats

Rat MSCs (rMSCs) were seeded in the porous HA hydrogels functionalized with peptide and cultured in osteogenic media for 1 week prior to implantation into the rat calvarial defects. After 12 weeks of implantation, micro computed tomography (micro CT) reconstruction reveals substantially more new bone formation in the defects implanted with the hydrogels conjugated with N-cadherin peptide (Cad + RGD) compared to the control groups (Blank, Scram + RGD, RGD) (Fig. 5A). Quantitative analysis shows that the relative new bone volume of the Cad + RGD group is significantly higher (Cad + RGD: 17.4%) than that of the control groups (Scram + RGD: 4.7%; RGD: 7.6%) (Fig. 5B). Both the hematoxylin/eosin staining (Fig. 5C) and the immunohistochemical staining against osteocalcin and Runx2 on the histological sections reveal elevated bone matrix deposition and increased expression of osteogenic markers in the Cad + RGD group compare to the Scram + RGD and RGD groups (Fig. S8). Additionally, more vessel-like structures (stained against CD31) are observed in the Cad + RGD group than in the control groups.

3. Discussion

In this study, we demonstrate that the HA hydrogels functionalized with the N-cadherin mimetic peptide promote the osteogenic differentiation of the seeded hMSCs by mimicking the pro-osteogenic niche in the endosteal space. This finding sheds light on the importance of the bio-functionalization of biomaterials for the applications in regenerative medicine. The developed bio-functionalized hydrogels can also be used as a platform to study the effect of microenvironment cues on osteogenesis.

N-cadherin regulates the osteogenesis primarily by mediating either the homotypic cell adhesion among MSCs or the “orthotypic” interaction between differentiated osteoblasts and MSCs [14]. The expression of N-cadherin increases in the early stage of

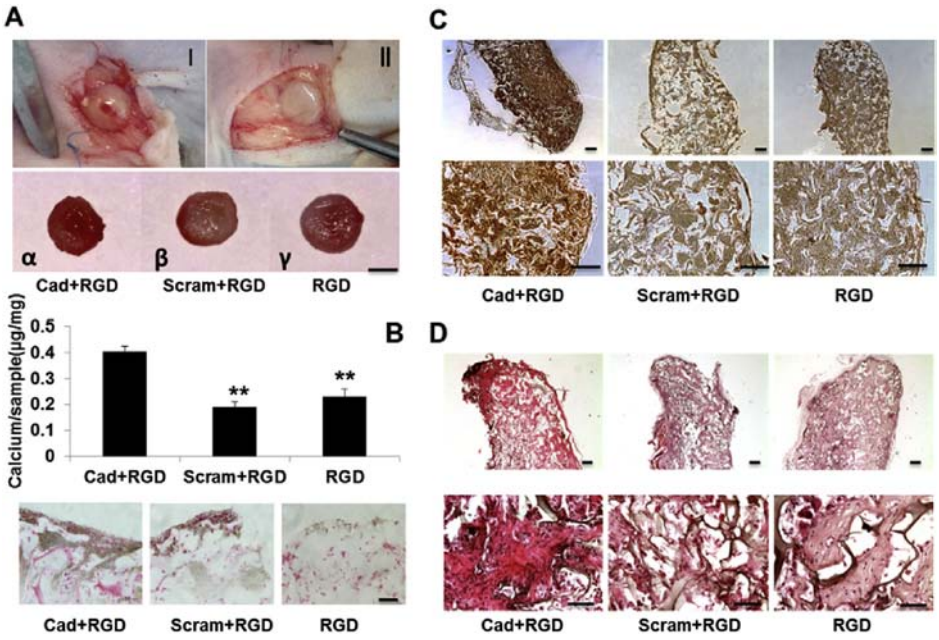

Fig. 4. Osteogenesis of the hMSCs seeded in N-cadherin peptide Bio-functionalized HA hydrogels in vivo. (A) Harvested hMSCs-laden 3D porous peptide-functionalized HA hydrogels after 28 days of subcutaneous implantation in nude mice (Ⅰ, α) Cad + RGD; (Ⅱ, β) Scram + RGD; (γ) RGD. Out of eight samples implanted per group, seven, seven and six samples are recovered from the Cad + RGD, Scram + RGD and RGD groups, respectively. (Scale bar = 2 mm) (B) Calcium content and Von Kossa staining of the implanted hydrogels after 28 days of subcutaneous implantation. (**$p < 0.01$ vs Cad + RGD, n = 3. Scale bar = 50 μm) (C) Immunohistochemical staining against type I collagen (Scale bar = 100 μm), and (D) Hematoxylin/eosin staining of the paraffin sections of the hMSCs-laden HA porous hydrogels after 28 days of implantation. (Scale bar: upper row = 100 μm; lower row = 25 μm).

osteoblastogenesis with increasing homotypic cell–cell contact and decreases in mature osteoblasts, which exhibit reduced cell–cell contact [4,12]. Our own work also shows a similar trend of decreasing N-cadherin expression in the hMSCs with increasing osteogenic culture time (Fig. S2). Previous studies suggest that N-cadherin-mediated homotypic cell adhesion promotes the initial osteogenic commitment of the MSCs, whereas the sustained over-expression of N-cadherin inhibits further differentiation into mature osteoblasts [15,16]. This negative effect of N-cadherin on mature osteoblasts is due to its interference with the canonical Wnt signaling, and it is mediated through membranous sequestration or the increased degradation of β-catenin, which is a key element in the canonical Wnt signaling pathways [17].

In this study, the low seeding density of the hMSCs (5000 cells/cm^2) on the 2D peptide functionalized HA hydrogel substrates and the slow proliferation of the hMSCs on the relatively soft HA hydrogels limit the extent of initial cell–cell interaction among the hMSCs. We believe that the interaction between the membranous N-cadherin of the hMSCs and the tethered N-cadherin peptide emulates the N-cadherin-mediated cell–cell interactions during the mesenchymal condensation, and therefore promotes the osteogenic differentiation of the hMSCs. Furthermore, more clustering of the seeded hMSCs is observed in the presence of the conjugated N-cadherin peptide (Cad + RGD group) (Fig. 2A). Therefore, the enhanced osteogenesis of the hMSCs in the Cad + RGD group may also be attributed to the increased cell–cell communication and the coordinated gene expression via gap junctions in the differentiating hMSCs because N-cadherin is found to be associated with connexin-mediated gap junction communications that promote osteogenesis. Because the hMSCs used in this study are not genetically manipulated to overexpress a sustained level of N-cadherin we speculate that the conjugated N-cadherin peptide (presented extracellularly to the hMSCs) is unlikely to substantially interfere with the Wnt signaling in the differentiating hMSCs.

N-cadherin also mediates the “orthotypic” interactions between differentiated osteoblasts and MSCs. The expression of a dominant-negative N-cadherin mutant or conditional knockout of N-cadherin in transgenic mouse osteoblasts leads to a significant reduction in adhesion between osteoblasts and bone marrow MSCs, thereby resulting in a severely reduced trabecular bone mass and a decreased bone formation rate [6,7]. In contrast, a recent study also shows that the overexpression of the N-cadherin in osteoblasts inhibits the osteogenesis of the bone marrow MSCs *in vivo* [18]. These findings indicate that on the one hand N-cadherin-mediated interaction between osteoblasts and bone marrow MSCs in the endosteal niche is crucial to the renewal and osteogenic commitment of bone marrow MSCs [7]. On the other hand, the over-expression of N-cadherin in the osteoblasts obstructs Wnt signaling, reduces the secretion of Wnt ligands of the osteoblasts, potentially restricts the escape of the MSCs for further differentiation, and thereby inhibits the osteogenesis of the MSCs [14,18,19]. Therefore, depending on its expression level, the N-cadherin in the osteoblasts seems to modulate a biphasic effect on the osteogenesis of the MSCs. Specifically, regular N-cadherin expression in the osteoblasts promotes the osteogenesis, whereas overexpression inhibits the osteogenesis of the MSCs.

In this study, the conjugation of the N-cadherin mimetic peptide imparts to the HA hydrogels the biological activity of N-cadherin, and this emulates the N-cadherins presented by the osteoblasts lining the trabecular surface (Fig. 1D). Therefore, we postulate that the “orthotypic” interaction between the hMSCs and the conjugated N-cadherin peptide recreates a biomimetic pro-osteogenic niche microenvironment in our 3D porous peptide-functionalized HA hydrogels, and contributes to the observed enhancement in the hMSC osteogenesis. Due to the degradation over time, the conjugated N-cadherin peptide is unlikely to impede further osteogenic differentiation of the hMSCs. The potential anti-osteogenesis effect of a high dose of the N-cadherin peptide will be investigated in future studies.

A previous study showed that the non-degradable nature of the

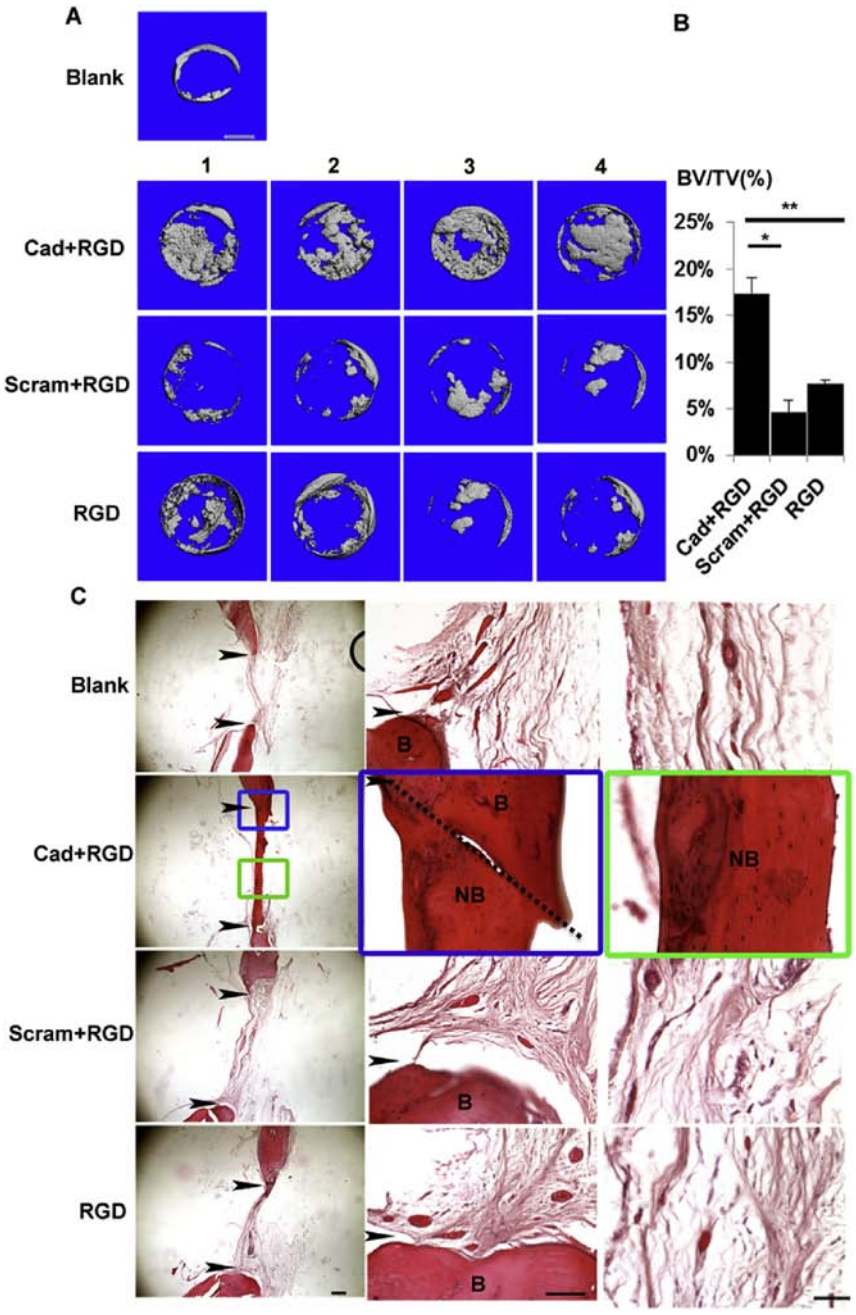

Fig. 5. Rat MSCs-laden porous hydrogels functionalized with N-cadherin peptide promote healing of calvarial defects in rats. (A) Micro CT reconstruction images, (Scale bar = 1 mm) (B) quantitative bone volume analysis (BV: bone volume; TV: total defect volume) and (C) hematoxylin and eosin staining images of SD rat calvaria defects after rMSCs encapsulated N-cadherin mimetic peptide conjugated 3D porous hydrogels (Cad + RGD) implanting for 12 weeks (black arrows: calvaria defect boundary; B: native bone; NB: newly formed bone; dotted line: interface between NB and B), with scrambled sequence and RGD peptide as control groups (Scram + RGD, RGD). (Scale bar: left column = 500 μm; middle column = 100 μm; right column = 50 μm).

solid methacrylated HA hydrogels inhibits the osteogenesis of the encapsulated hMSCs by restricting cell spreading and cell traction force development even in the presence of RGD [20]. Therefore, in this study, hMSCs were seeded either on top of the flat HA hydrogel substrates or the internal pore surfaces of the porous 3D HA hydrogels. Hence, the RGD peptide is conjugated to the HA hydrogels in addition to the N-cadherin peptide in all groups to enable cell adhesion because the N-cadherin peptide used in this study alone does not support cell adhesion on HA hydrogels. The pro-osteogenic effect of the N-cadherin peptide is therefore not investigated in the absence of the RGD peptide. This is a limitation of this study. Meanwhile, the integrins that bind to the RGD peptide are known to be important to the osteogenic differentiation of MSCs [21,22]. Therefore, the presence of the RGD peptide may further complement the artificial pro-osteogenic niche in our 3D porous hydrogels contributing to the enhanced osteogenesis of the hMSCs. In view of the reported crosstalk actions between integrin and cadherin signaling, future investigations will examine the potential synergistic effect of presenting N-cadherin peptide together with the RGD peptide [23].

Cadherins, including N-cadherins, interact with actin structures via adaptor proteins, such as α-catenin, and they are involved in

mechanotransduction via actomyosin contraction [24–26]. It should be noted that previous studies have also shown that the direct addition of free diffusing N-cadherin mimetic peptide into the culture media not only decreases bone nodule formation by primary calvaria cells but also inhibits cell–cell interaction and the osteogenesis of bone marrow MSCs [4,5]. In our study, direct supplementation of the free unconjugated N-cadherin mimetic peptide, but not the control peptide of the scrambled sequence, significantly reduces the ALP activity of the hMSCs (Fig. S5). This is likely due to the fact that the untethered peptides function as antagonists to the membranous N-cadherin by competitive binding, which impairs the homotypic binding of native N-cadherins on neighboring cells and the resulting intracellular signaling events. In contrast, the conjugation of the N-cadherin peptide to the HA hydrogels promotes cell–cell clustering and the osteogenic differentiation of the attached hMSCs. We postulate that the binding between the native membranous N-cadherin of the hMSC and the N-cadherin peptide conjugated on the HA hydrogels likely better mimics the mechanosensing of the homotypic interaction of the native N-cadherins, and that it is capable of initiating the downstream signaling events required for hMSC lineage specification.

The differentiation of the hMSCs has been shown to be regulated by the substrate stiffness [27]. It should be noted that the HA hydrogels used in this study are much softer than the tissue culture plastics (TCPS), which are generally used for studying the osteogenesis of hMSCs. The Young's moduli of the HA hydrogels are around 10–20 kPa, and they are nonetheless sufficiently stiff to support the osteogenesis of hMSCs [27]. The soft hydrogels are used to slow down the proliferation and differentiation of the hMSCs in order to prevent the early confluence of the seeded hMSCs. This makes the long-term investigation on the effect of N-cadherin peptide possible without using the antimitotic drugs, which may introduce additional confounding factors. The late detection of the difference in the ALP activities, an early osteogenic marker, between the Cad + RGD and the control groups may also be due to the extended differentiation on the soft hydrogels. In addition, RGD peptides are used in this study to provide adhesion sites for the seeded hMSCs because, based on our preliminary study, hMSCs do not adhere to HA hydrogels conjugated with N-cadherin peptide alone. The conjugation of the N-cadherin peptide in the Cad + RGD group consumes only an additional 10% of the total methacrylates in the MeHA macromer compared to the RGD only control group. Therefore, the difference in the mechanical stiffness of the HA hydrogels due to varying methacrylate consumptions for the peptide conjugation between different groups is negligible, and it is unlikely to significantly impact the differentiation of the seeded hMSCs.

In this study, HA hydrogels are used as the substrate material to present the N-cadherin mimetic peptide because of the pro-osteogenic property of HA. Moreover, HA hydrogels are non-fouling materials on which non-specific adhesions of serum proteins from the culture media are minimized [28,29]. Therefore, the HA hydrogels provide a “blank slate” for ascertaining the effect of the conjugated peptides on hMSC adhesion and differentiation free from the interference of other cell adhesion proteins in the media. In addition, HA has been shown to augment the integrin-mediated mechanotransduction indicating crosstalks between HA receptors and specific integrins [30]. Whether the enhanced osteogenesis of the hMSCs seeded on the HA hydrogels conjugated with N-cadherin peptide can be attributed to potential interactions between HA receptors and N-cadherin certainly warrants further investigation. We are currently planning to evaluate the effect of N-cadherin peptide on the differentiation of hMSCs by using biopolymers other than HA as the substrate.

4. Materials and methods

4.1. Macromer synthesis and hydrogel preparation

Methacrylated HA (MeHA) was synthesized with sodium hyaluronate powder (MW ~ 74 kDa, Lifecore), as previously reported [31]. MeHA with a methacrylation level of either ~30% or ~100%, as confirmed by ^{1}H NMR, was synthesized. N-cadherin (Ac-HAVDIGGGC) mimetic or scrambled sequence (Ac-AGVGDHIGC) peptide and RGD (GCGYGRGDSPG) peptide (GenScript) with a cysteine residue at the C-terminal end were conjugated on MeHA backbone by Michael-type addition reaction with the methacrylate groups in alkaline phosphate buffer (0.2 M Na_2HPO_4–NaH_2PO_4, pH 8.0) at 37 °C overnight. 2D peptide functionalized HA hydrogel substrates were fabricated by polymerizing peptide-conjugated MeHA precursor solutions (3% w/v, 30% methacrylation) under ultraviolet light (UV, wavelength: 365 nm; intensity:7 mW/cm^2 time:30 min) on the methacrylated glass coverslips (Fig. 1A). 0.05% (w/v) of 2-methyl-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (I2959, Ciba) was used as the photoinitiator. 3D porous peptide functionalized HA (3% w/v, ~100% methacrylation) hydrogels were formed by curing precursor solutions with dithiothreitol (DTT) as the crosslinker in PVC molds packed with PMMA microsphere porogens (Ø250 μm) (Fig. 1BCD). The obtained constructs were sterilized by 75% ethanol and rinsed with de-ionized water after PMMA microspheres were leached out in acetone overnight.

4.2. Cell culture and seeding on hydrogels

Human MSCs (Lonza) were expanded to passage 4 in growth media containing α-MEM with 16.7% FBS, 1% glutamine and 1% pen/strep. hMSCs (5,000 cells/cm^2) were cultured on the 2D peptide functionalized HA hydrogel substrates (Ø15.6 mm, 0.5 mm thickness; Fig. 2B), which were conjugated with N-cadherin mimetic peptide and RGD peptide (Cad + RGD), scrambled sequence peptide and RGD peptide (Scram + RGD), or with RGD peptide (RGD) alone. The molar ratio between the methacrylate groups and the peptide thiols is 10 (methacrylate): 1(peptide thiol). MSCs (20 million/ml, 50 μl) were seeded into 3D porous peptide functionalized HA hydrogels (the same grouping and peptide conjugation ratio as that in the 2D hydrogels) for 2 h for cell attachment before further cell culturing. Constructs were then cultured in osteogenic media (α-MEM, 16.67% FBS, 1% glutamine, 10 mM β-glycerophosphate disodium, 50 μg/ml ascorbate, 100 nM dexamethasone), which was changed three times per week, and evaluated at selected time points. Cell viability was assessed by using a Live/Dead assay, in which live cells are stained green with Calcein-AM and dead cells are stained red with ethidium bromide.

4.3. Staining of the biological markers on 2D hydrogel substrates

Human MSCs cultured on the 2D peptide functionalized HA hydrogel substrates were fixed in 4% paraform for 10 min, rinsed in PBS for several times, and permeabilized with 0.25% TritonX-100 in PBS for 30 min at room temperature. Alkaline phosphatase activity was stained by Fast Blue staining (Sigma). Tissue calcification was stained by Von Kossa staining. For immunofluorescence staining, samples were blocked with 0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 min at 37 °C after permeabilization. Samples were incubated with primary antibodies (1:100 dilution) in 0.5% BSA at 4 °C overnight, and subsequently with secondary antibodies (1:200 dilution) in blocking solution for 2 h at room temperature. To estimate the Runx2 expression, Image J was used to measure the ratio between the nuclear and cytoplasmic fluorescence intensity of the hMSCs stained with a Runx2 antibody

(mouse monoclonal anti-Runx2, Santa Cruz).

4.4. Subcutaneous implantation in nude mice

Human MSCs-laden (1 million cells/hydrogel) 3D porous peptide functionalized HA hydrogels (n = 8 for each group) were fabricated as described above and cultured in osteogenic media for one week before implantation. Four subcutaneous pockets on the back of each nude mouse were prepared for implantation (age 8–10 weeks). Samples were harvested after four weeks of *in vivo* implantation. Guidelines from the Institutional Animal Care and Use Committee at The Chinese University of Hong Kong were followed during all animal procedures.

4.5. Evaluation in rat calvarial bone defects

Male and mature Sprague Dawley (SD) rats (12weeks old) were clipped and prepped for aseptic surgery following the guidelines from the Institutional Animal Care and Use Committee at The Chinese University of Hong Kong. A midline skin flap was raised over the parietal bones and reflected caudally to expose the midsaggital and transverse sutures. Periosteum was incised along the mid-saggital suture and the right or left transverse suture, and removed to expose the parietal bone. A 5 mm diameter defect was created using a trephine with normal saline irrigation during processing, and the section of bone was removed to expose the dura mater [32]. 5 mm diameter and 1 mm thick 3D porous peptide functionalized HA hydrogels (n = 4 for each group) were seeded with rMSCs (rat mesenchymal stem cells, 1 million cells/hydrogel). After 7 days of *in vitro* osteogenic differentiation, the hydrogels were implanted into the calvarial defects. All experimental animals were maintained until 12 weeks from the day of defect creation. After euthanizing, the parietal bones were harvested and decalcified with 10% EDTA solution. The histological analysis and the following hematoxylin/eosin staining were done as described before.

4.6. Statistical analysis

All data are presented as mean ± standard deviation. Statistical analysis was performed by using two-way ANOVA and Tukey's HSD post hoc testing to allow comparison between groups with experimental group as the independent factor (sample sizes are provided in the figure captions).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.10.072.

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