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Self-assembled N-cadherin mimetic peptide hydrogels promote the chondrogenesis of mesenchymal stem cells through inhibition of canonical Wnt/β-catenin signaling

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ABSTRACT

N-cadherin, a transmembrane protein and major component of adherens junction, mediates cell-cell interactions and intracellular signaling that are important to the regulation of cell behaviors and organ development. Previous studies have identified mimetic peptides that possess similar bioactivity as that of N-cadherin, which promotes chondrogenesis of human mesenchymal stem cells (hMSCs); however, the molecular mechanism remains unknown. In this study, we combined the N-cadherin mimetic peptide (HAVDI) with the self-assembling KLD-12 peptide: the resultant peptide is capable of self-assembling into hydrogels functionalized with N-cadherin peptide in phosphate-buffered saline (PBS) at 37 °C. Encapsulation of hMSCs in these hydrogels showed enhanced expression of chondrogenic marker genes and deposition of cartilage specific extracellular matrix rich in proteoglycan and Type II Collagen compared to control hydrogels, with a scrambled-sequence peptide after 14 days of chondrogenic culture. Furthermore, western blot showed a significantly higher expression of active glycogen synthase kinase-3β (GSK-3β), which phosphorylates β-catenin and facilitates ubiquitin-mediated degradation, as well as a lower expression of β-catenin and LEF1 in the N-cadherin peptide hydrogels versus controls. Immunofluorescence staining revealed significantly less nuclear localization of β-catenin in N-cadherin mimetic peptide hydrogels. Our findings suggest that N-cadherin peptide hydrogels suppress canonical Wnt signaling in hMSCs by reducing β-catenin nuclear translocation and the associated transcriptional activity of β-catenin/LEF-1/TCF complex, thereby enhancing the chondrogenesis of hMSCs. Our biomimetic self-assembled peptide hydrogels can serve as a tailorable and versatile three-dimensional culture platform to investigate the effect of biofunctionalization on stem cell behavior.

1. Introduction

N-cadherin plays an important role in mediating cell–cell interactions during mesenchymal condensation, which is critical to the development and formation of multiple musculoskeletal tissues including articular cartilage [1–3]. The expression of the dominant negative form of N-cadherin or treatment with N-cadherin antibody leads to inferior hyaline cartilage development *in vivo* [4,5]. Our early work demonstrated that the conjugation of a N-cadherin mimetic peptide containing the "HAVDI" sequence derived from the first extracellular domain of N-cadherin emulates the cell-cell interaction cue and enhances the chondrogenesis of

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encapsulated human mesenchymal stem cells (hMSCs) in hyaluronic acid hydrogels [6]. This finding highlights the importance of the biofunctionalization of biomaterial scaffolds, with developmentally relevant cues in enhancing the regenerative outcome of stem cell-based treatments. Nevertheless, the molecular mechanism of the pro-chondrogenic effect of N-cadherin mimetic peptide remains elusive.

To investigate the molecular mechanism of N-cadherin mimetic peptide promoting chondrogenesis, it is necessary to decouple the inductive effects of the hyaluronic acid hydrogels used in our earlier study because hyaluronic acid is known to interact with hMSCs through pro-chondrogenesis receptors including CD44 and CD168 [7]. The self-assembly peptide (SAP) formed hydrogels are emerging biomaterials for cartilage regeneration because the formation of these hydrogels is driven by spontaneous self-assembly of peptides in the absence of chemical crosslinkers, or other stimulation such as heating, light exposure or pH change. Thereby, this affords excellent cytocompatibility in these hydrogels, avoiding potentially harmful UV exposure in photo-crosslinking [8–16]. One of the SAPs, KLD peptide, is composed of repeating alternative sequence of lysine (K), leucine (L), and aspartic acid (D) [10,17,18]. Under the shielding effects of the Phosphate-buffered saline (PBS) aqueous environment, single peptide segments were clustered under the hydrophobicity of leucine; and nanofibers were extended under the electrostatic force between positive charges form lysine and negative charges from aspartic acid, resembling other amphiphilic self-assembled peptide fibers [19,20]. Nanofibers finally bundle to form a free-standing hydrogel. The self-assembled peptide nanofibers resemble the fibrous cartilaginous extracellular matrix and provide a conducive three dimensional (3D) microenvironment with biomimetic nanoscale architecture for the proliferation and chondrogenic differentiation of hMSCs [12,21–23]. Studies have shown that SAP hydrogels support chondrogenesis of the encapsulated MSCs and facilitate cartilage repair *in vivo* by mediating controlled delivery of TGF-β [17,24–27]. Furthermore, appending bioactive peptide sequences to the self-assembled peptide also allows their facile incorporation and affords additional bioactivity in the formed hydrogels [20,28–32]. However, being encapsulated or embedded in isolation, MSCs in such hydrogels have limited cell–cell interactions during the early stage of differentiation induction; this may hamper the chondrogenesis and the osteogenesis of MSCs. Many researchers tethered the extracellular matrix component, growth factors, and other bioactive components for better biomaterial functioning [33–37]. Our early works demonstrated that the conjugation of N-cadherin mimetic peptide in photocrosslinked solid hydrogels promoted the chondrogenesis of encapsulated hMSCs with round morphology in the presence of soluble chondrogenic factor (TGF-β3), whereas the conjugation of N-cadherin peptide in chemically-crosslinked porous hydrogels enhanced the osteogenesis of seeded hMSCs with spread morphology in the presence of soluble osteogenic factor (dexamethasone) [6,37]. Our collective findings suggest that the lineage-specific pro-differentiation effect of conjugated N-cadherin mimetic peptide is dependent on the concerted actions of other microenvironmental cues including the inductive soluble factors in the culture media and hydrogel structures that dictate cell spreading and mechanosensing on the surrounding matrix. However, the photocrosslinked and chemically-crosslinked hydrogels face major hurdles in clinical translation due to the potential cytotoxicity of the UV light source and free radicals generated that are required for hydrogel fabrication. This motivates us to incorporate the N-cadherin mimetic peptide into the self-assembled peptide hydrogels to study the molecular mechanism of N-cadherin peptide in promoting chondrogenesis of encapsulated hMSCs in the absence of cytotoxic factors.

In this study, we designed self-assembled peptide hydrogels that are functionalized with N-cadherin mimetic peptide, to examine the signaling mechanism of enhanced chondrogenesis by this mimetic peptide. The incorporation of the N-cadherin peptide in the self-assembled peptide hydrogels significantly promotes the expression of chondrogenic markers and cartilaginous matrix production by encapsulated hMSCs. We hypothesize that N-cadherin mimetic peptide promotes hMSC chondrogenesis by inhibiting the transcription of canonical Wnt signaling. Corroborating this hypothesis, our results from RT-qPCR, western blotting, and immunofluorescence staining show that the conjugated N-cadherin peptide increases the expression of GSK-3β and decreases the nuclear localization of β-catenin. We believe that this study sheds light to the underlying molecular mechanism of the pro-chondrogenic effect of N-cadherin peptide in the context of chondrogenic inductive condition. This finding will aid in further optimization of biofunctionalized biomaterials to support stem cell-based cartilage repair and regeneration.

2. Methods

2.1. Peptide synthesis and self-assembled hydrogel fabrication

Self-assembly peptide (Ac-KLDLKLDLKLDL, KLD in short), N-cadherin mimetic self-assembly peptide (Ac-HAVDIGGKLDLKLDLKLDL, KLD-Cad in short), and scrambled control peptide (Ac-AGVIDHGKLDLKLDLKLDL, KLD-Scr in short) were synthesized by Genescript (Nanjing, China). The purification of peptide powders were 90.52%, 92.51%, and 92.40%, confirmed by high performance liquid chromatography. Mass spectrometry results assured the correct molecular weight of these three kinds of peptides (Figs. S1, S2, S3). To ensure sterilization, we first UV sterilized all the solid materials (peptide, molds, NaOH) for over 1 h in biosafety level II hood, and used autoclaved DI water and PBS in hydrogel fabrication and cell encapsulation process. All solutions for peptide dissolution and hydrogel fabrication were also filtered with 0.2 μM nylon filter before use.

KLD, KLD-Cad, and KLD-Scr peptide powders were separately dissolved in sterilized 0.1 mM NaOH solution (1%, w/v), and sonicated for 30 min prior to use. To prepare the KLD hydrogel, KLD solution was diluted with sterilized phosphate buffer saline (PBS) to a final peptide concentration of 0.5% (w/v). For the KLD-Cad hydrogel and the KLD-Scr hydrogel, we first mixed 1% (w/v) KLD solution with 1% (w/v) KLD-Cad or KLD-Scr solution at volume ratio of 1:1, then diluted the precursor mixture to 0.5% (w/v) with sterilized PBS. After filling the mold (Ø 4.71 mm, 5 mm thickness), the entire construct was maintained at 37 °C for at least 20 min for gelation. When mixing precursor solution with hMSCs, the gelation time was extended to 30 min to ensure the mechanical strength of the hydrogel. Hydrogels with a defined shape could then be observed and transferred to chondrogenic culture media.

Rheological test of self-assembled hydrogel on mechanical property with time sweep.

Mechanical properties of the hydrogels were measured by a Malvern Kinexus Rotational Rheometer (Malvern, Worcestershire, UK) equipped with a 10-mm diameter indenter and a 1.36 mm truncation gap. For time sweep experiments, the total testing time was 2 min per test, with a constant strain of 40% and fixed frequency of 1 Hz. In brief, each prepared KLD, KLD-Cad, and KLD-Scr hydrogel ($d = 10$ mm, $h = 2.26$ mm) was placed on the rheometer plate, and the indenter pressed on samples to truncation gap height. After equilibrating for 5 min, the time sweep tests of storage (G') and loss (G'') modulus were measured at room temperature (25 °C) at a frequency of 1 rad/sec.