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Single Cell Imaging to Probe Mesenchymal Stem Cell N-Cadherin Mediated Signaling within Hydrogels

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Abstract

N-cadherin (Ncad) mediates cell-cell interactions, regulates β -catenin (β cat) signaling, and promotes the chondrogenic differentiation of mesenchymal lineage cells. Here, we utilized confocal imaging to investigate the influence of Ncad interactions on single mesenchymal stem cell (MSC) behavior within 3-dimensional hydrogel environments under conditions that promote chondrogenic differentiation. Human MSCs were photoencapsulated in hyaluronic acid (HA) hydrogels functionalized with Ncad mimetic peptides and compared to cells in environments with control non-active peptides (Ctrl). Using single-cell imaging, we observed a significant increase in membrane β cat, nuclear β cat, and cell roundness after 3 days in Ncad hydrogels compared to Ctrl hydrogels. The extent of membrane and nuclear β cat localization and MSC roundness decreased to Ctrl hydrogel levels via pre-treatment with Ncad-specific antibodies prior to encapsulation in the Ncad hydrogels, confirming the activity of the peptide. Interestingly, there was a pronounced (>80% increase) in β cat nuclear localization in two-cell clusters within the Ctrl hydrogels, which was much greater than the increase (~30%) in β cat nuclear localization in two-cell clusters within the Ncad hydrogels. In summary, we utilized fluorescent imaging to demonstrate Ncad-mediated single cell responses to developmental cues within hydrogels towards chondrogenesis.

Keywords

Hyaluronic acid; N-cadherin; Biomaterials; Tissue Engineering

Introduction

Due to limitations in the natural healing capacity of cartilage and given the increasing incidence of osteoarthritis, there exists a growing demand for cell-based therapies for cartilage repair. Tissue engineering, and particularly approaches based on autologous mesenchymal stem cells (MSCs), has evolved to yield clinically relevant techniques that are translating into patient therapies.¹⁹ To increase the clinical efficacy of these approaches, it is important to understand the interface between MSCs and their microenvironment, particularly as it is becoming clear that biochemical and biophysical signals within stem cell environments can play an important role in controlling differentiation.¹⁰ For example, the extent of chondrogenesis within hydrogels can be regulated by signals that mimic components of native cartilage, either during development or within adult tissues.^{7,16}

Towards stem-cell based cartilage tissue engineering, we have used hydrogels formed from hyaluronic acid (HA) precursors, due to the role that HA has on cell behavior via receptor interactions (i.e., CD44, CD168),^{4,6,17} including during development.¹⁵ Beyond interactions with HA, other features of the microenvironment are important, including cell-cell interactions. N-cadherin (Ncad), a transmembrane protein that mediates cell-cell adhesion, is widely considered to be a key factor in directing cell-cell interactions during mesenchymal condensation.^{1,22,29} Indeed, overexpression of Ncad in the developing limb bud promotes early chondrogenic differentiation of the mesenchyme, while expression of Ncad dominant negative isoforms delays or prevents this differentiation lineage. Despite the importance of such interactions, most hydrogel-based approaches encapsulate single MSCs that are separated from one another by the biomaterial and inherently limit cell-cell contact. The His-Ala-Val (HAV) motif in the first extracellular domain of Ncad was previously identified as a critical regulator of cell-cell interactions,⁵ and we have shown that the incorporation of an HAV motif into HA hydrogels results in both an upregulation of chondrogenic genes within the first 3 days of culture and enhanced matrix production by photoencapsulated MSCs.⁴

Importantly, this study identified Ncad peptides as a potentially important target for the engineering of hydrogels for cartilage repair; however, additional information is needed regarding these interactions between Ncad peptide mimics presented in the hydrogel and encapsulated MSCs. For example, it is well known that β -catenin (β cat) plays a central role in both cell-cell junctions and Wnt signaling.¹⁴ Upon engagement of the extracellular domain of Ncad, the cytoplasmic domain of Ncad forms complexes with β cat, recruiting β cat to the membrane and reducing β cat phosphorylation, which leads to ubiquitin-dependent degradation.²⁵ Membrane β cat recruitment also results in an increase of available β cat in the cell, which permits β cat localization to the nucleus and the activation of numerous pathways. Notably, several studies have reported that cells in densely populated environments that recapitulate mesenchymal condensation exhibit an increase in nuclear β cat. For example, Tuli et al. showed that pellet cultures in culture media supplemented with TGF- β exhibited an increase in nuclear β cat.³⁰ Additionally, Modarresi et al. reported that overexpression of Ncad in micromass cultures resulted in an increase of nuclear β cat localization.²¹ Taken together, the presence of β cat in both the membrane and in the nucleus indicates cellular Ncad engagement with the Ncad peptides presented in the hydrogels.

To observe such interactions, techniques must be used that probe outcomes in single cells, rather than total population analyses. Additionally, it is well understood that MSC cultures are innately heterogeneous and variant cell behavior and differentiation potential can be observed across cells within the same cultures.^{3,24} For instance, after 7 days in culture, bovine MSCs photoencapsulated in hydrogels supplemented with chondrogenic induction media displayed variable proteoglycan production, indicating that some cells are more efficient at secreting matrix than others under identical microenvironments.^{8,12} Again, imaging techniques are needed to understand cell signaling in the context of MSC heterogeneity within hydrogels.

We hypothesize that the introduction of Ncad mimetic peptides (i.e., HAV) into hydrogels will increase both membrane and nuclear β cat. To assess Ncad-mediated signaling in the

context of membrane and nuclear β cat localization, MSCs were photoencapsulated in HA hydrogels from methacrylated HA (MeHA) macromers functionalized with either Ncad mimetic (Ncad) or non-active control peptides (Ctrl) (Fig. 1). Confocal imaging was then utilized to assess membrane β cat, cell morphology, and nuclear β cat (defined as the volume ratio of β cat in the nucleus to that of the cytosol). Furthermore, this approach allowed us to compare single cells versus two-cell clusters within hydrogels, which would not be possible with population-based measurement techniques.

Materials and Methods

MeHA synthesis and peptide coupling

MeHA was synthesized as previously described.²⁸ Briefly, methacrylic anhydride was reacted with 1% (wt/v) sodium hyaluronate powder (molecular weight ~75 kDa, Lifecore) in deionized water at a pH of 8. Next, MeHA was purified via dialysis (SpectraPor, 6-8 kDa molecular weight cutoff) for 4 days and lyophilized for at least 3 days. The extent of MeHA methacrylation was confirmed by ¹H NMR to be ~29%. Ncad (Ac-HAVDIGGGC) and Ctrl (Ac-AGVGDHIGC) peptides (827.9 Da) with a cysteine residue at the C-terminal end were obtained from GenScript. For Michael-type addition peptide coupling, 9.28 mg of peptide and 100 mg MeHA were dissolved in triethanolamine-buffered saline (TEOA buffer, pH 8) and reacted at 37 °C overnight, followed by dialysis (4 days) and lyophilization (3 days). This coupling ratio was used to obtain a final concentration of peptide of 2 mM in a 3 wt% hydrogel.

Cell culture and hydrogel encapsulation

Human MSCs (Lonza) expanded to passage 3 in growth media (α -MEM supplemented with 16.7% (v/v) FBS (Gibco), 1% (v/v) L-glutamine (Invitrogen), 1% (v/v) penicillin-streptomycin (Invitrogen)) were used in all experiments. For Ncad blocking studies, 2.5 million MSCs were suspended in 2 mL buffer (2% (v/v) FBS in PBS) or 2 mL anti-Ncad antibody in buffer (50 μ g/mL, clone GC-4, Sigma) for 45 min on ice, followed by two centrifugation washes (500 RCF) in buffer and resuspension in PBS prior to photoencapsulation.

HA hydrogels containing MSCs (5 million cells/mL) were formed by exposing MeHA macromer solutions (3 wt%) to light (365 nm, 2 mW/cm², 10 min) in the presence of 0.05% (w/v) photoinitiator (I2959 Irgacure, Ciba). Hydrogel disks (5 mm diameter, 2.6 mm thickness) contained either the Ncad mimetic peptide (Ncad) or the scrambled non-active control peptide (Ctrl). MSC-laden hydrogels were cultured in chondrogenic media (α -MEM, 1% (v/v) ITS+Premix, 50 μ g/ml L-proline, 0.1 μ M dexamethasone, 0.9 mM sodium pyruvate, 50 μ g/ml ascorbate, 1% (v/v) penicillin-streptomycin) supplemented with 10 ng/ml TGF- β 3 for 3 days.

Immunostaining and imaging

After 3 days in culture, MSC-laden hydrogels were fixed in 10% formalin overnight at 4 °C. Samples were then permeabilized with 0.1% Triton X-100 (30 min) and blocked with 3% normal goat serum (1 hour) in PBS. Primary β cat antibody (Abcam, 1:100) was added

overnight at 4 °C, followed by Alexa Fluor 488 secondary antibody (Life Technologies, 1:200) for 2 hours at room temperature. To visualize individual cells and nuclei, samples were stained for actin (rhodamine phalloidin, 1 hour, 1:100) and double-stranded DNA (DAPI, 30 min, 1:500), respectively. After staining, samples were washed and imaged with a 20X objective using a Leica SP8 inverted confocal microscope. Using this setup, 3-dimensional image stacks of xy-plane cross-sections were acquired (thickness ~150 µm, step-size 0.69 µm). To confirm MSC viability under these conditions, a live/dead assay was used per the manufacturer's instructions (Life Technologies). Briefly, this assay simultaneously stains cells with green-fluorescent calcein-AM in viable cells and red-fluorescent ethidium homodimer in compromised cells to indicate intracellular esterase activity and loss of plasma membrane integrity, respectively.

Image Analysis

All image analysis was performed using ImageJ NIH image processing software (Bethesda, MD). To quantify membrane β cat, cross-sectional images at the centroid from 3-dimensional β cat channel image stacks were used to calculate horizontal average pixel intensity profiles of two-pixel thick rectangles spanning the length of the cell (Fig. 2A). To account for differences in cell size, horizontal distances were normalized so they range from 0 to 1, and pixel intensity values were subtracted and divided by the average pixel intensity of the rectangles to account for cell-to-cell variability in total pixel intensity.

For each cell, the nuclear and cytosolic domains were determined by generating binary masks of 3-dimensional image stacks of double-stranded DNA and actin using Otsu's intensity-based thresholding method (Fig. 3A).²³ Actin staining was only used to assess cellular morphology and not actin distribution throughout the cells. Image J's 3D Objects Counter function was then used to calculate volume and surface area for each domain. These values were used to define cellular roundness by determining the ratio between the cell's surface area divided by the surface area of a sphere with the cell's volume, resulting in a value ranging from 0 to 1, where 0 is a straight line and 1 is a perfect sphere.

To quantify nuclear β cat, nuclei masks were inverted and superimposed with corresponding actin masks to generate masks that encompass the cytosol yet exclude the nucleus. 3-dimensional β cat image stacks of the same cells were then superimposed with these masks, resulting in new image stacks containing cytosolic β cat. Similar methods were employed using nuclei masks to obtain nuclear β cat image stacks. These image stacks were then subject to Image J's Plot Z-axis Profile function to calculate total pixel intensity for each cross-section in the 3-dimensional image stacks. Using this data, the ratio between nuclear and cytosolic β cat was calculated and normalized to the volumes of the nuclear and cytosolic domains within the cell (Fig. 3A). For multiple cells (two-cell clusters), the same approach for determining nuclear β cat was utilized with multiple nuclei.

Statistical analysis

Statistical analyses were performed with one-way ANOVA with Tukey's *post hoc* test using Excel software with Daniel's XL Toolbox add-in. Differences between culture conditions

were considered significant for p-values less than 0.01, and error bars indicate standard mean error around the mean. At least 30 cells were analyzed per group and condition.

Results

Ncad mimetic peptides recruit β cat to the cell membrane

HA hydrogels were formulated from MeHA macromers that contained covalently bound peptide - either HAV, which was identified from the extracellular domain of Ncad (Ncad), or the same sequence scrambled into a non-active form (Ctrl). To confirm cell viability when MSCs were photoencapsulated in 3 wt% hydrogels containing either peptide, a live/dead assay was performed after 3 days. High viability (>95%) was observed across all hydrogel formulations with no noticeable differences between groups (Fig. 1C). To assess membrane β cat differences of MSCs in Ncad and Ctrl hydrogels, β cat cross-sections were first taken at the centroid of single MSCs (Fig. 2A). Next, horizontal average intensity profiles were obtained from these cross-sections, in a two-pixel thick rectangle that crossed the centroid and spanned the length of the cell. Prior to plotting, distance and pixel intensity were normalized, such that distance ranged from 0 (left side of rectangle) to 1 (opposite end), and pixel values were subtracted and divided by the average pixel intensity of the rectangle to account for cell-to-cell variability in pixel intensity.

Representative β cat images of single MSCs in Ncad hydrogels (Fig. 2B, **left collage**) featured more membrane β cat than those in the Ctrl hydrogels (Fig. 2B, **center collage**). A normalized average pixel intensity versus horizontal distance plot of single MSCs in Ncad hydrogels (gray lines denote single cell profiles and thick red line denotes population average) features intensity peaks near the ends of the graph, which correspond to the cell periphery (Fig. 2C, **top plot**). In contrast, the images and plot of MSCs in the Ctrl group (thick blue line denotes population average) do not display regions of high intensity and β cat was fairly uniform throughout the span of the cell (Fig. 2C, **bottom plot**).

Ncad mimetic peptides enhance MSC roundness and nuclear β cat localization

To assess the influence of Ncad peptides on cellular roundness and nuclear β cat, single MSCs were stained for β cat, actin, and double-stranded DNA, and Otsu's intensity-based thresholding method was used to generate 3-dimensional masks of the cell (V_{cell}) and nucleus (V_{nuc}) (Fig. 3A). Cell volume and surface area were calculated using cell masks, and these values were used to calculate cell roundness, which is the surface area ratio between the cell and a sphere with the same volume as the cell. To create cytosol masks that include the cell body and exclude the nuclear domain (V_{cyt}), nuclei masks were inverted and superimposed with corresponding cell masks. 3-dimensional β cat image stacks were then superimposed with V_{nuc} and V_{cyt} to obtain image stacks of β cat pixel intensity in the nuclear ($\beta\text{cat}_{\text{nuc}}$) and cytosolic ($\beta\text{cat}_{\text{cyt}}$) space. Nuclear localization of β cat (nuclear β cat) was determined by taking the ratio between total $\beta\text{cat}_{\text{nuc}}$ and $\beta\text{cat}_{\text{cyt}}$ pixel intensities normalized to V_{nuc} and V_{cyt} volumes, respectively.

Representative actin and nuclei images of single MSCs in the Ncad (Fig. 3B **left**) and Ctrl (Fig. 3B **center**) conditions showed that cells were similar in size, but exhibited differences

in cellular morphologies between groups. In the Ncad condition there was generally more membrane and nuclear β cat compared to the Ctrl hydrogels, which did not contain the active Ncad mimetic peptides (Fig. 3C **left and center**).

Roundness was significantly lower for MSCs in the Ctrl condition, suggesting some degree of localized cell spreading into the hydrogel (Fig. 4A). Likewise, nuclear β cat was significantly lower in the Ctrl condition (Fig. 4B). To assess nuclear β cat population heterogeneity of MSCs in Ncad hydrogels, a nuclear β cat histogram was plotted (Fig. 4C). Correlations between cell morphology and nuclear β cat localization were also determined by plotting nuclear β cat versus roundness (Fig. 4D). In both cases, these plots showed that single cells in the Ctrl condition formed a cluster that was distinguishable from the cells in the Ncad hydrogels, and that both groups exhibited heterogeneity.

Blocking Ncad transmembrane proteins mitigates effects of Ncad mimetic peptides

To ascertain whether cell-biomaterial interactions between Ncad transmembrane proteins on cells and Ncad peptides in hydrogels led to changes in β cat membrane/nuclear translocation and roundness, MSCs were treated with a monoclonal Ncad antibody (Ab+) prior to photoencapsulation in Ncad hydrogels (Fig. 1B) and remained highly viable for the duration of the study (Fig. 1C). By doing so, a comparison could be made between untreated MSCs in Ncad hydrogels which were able to interact with Ncad peptides in the hydrogel and MSCs in the Ab+ group that were unable to interact with the Ncad peptides due to Ncad blocking.

Representative β cat images of MSCs in the Ab+ group (Fig. 2B, **right collage**) feature significantly less membrane β cat than untreated MSCs in the same Ncad formulation (Fig. 2B, **left collage**). A normalized pixel intensity versus horizontal distance plot of the Ab+ group (gray lines denote single cell profiles and thick green line denotes population average) shows a uniform pixel intensity distribution throughout the cells (Fig. 2D), which contrasts observations seen in untreated MSCs in Ncad hydrogels (Fig. 2C, **top**), yet is similar to MSCs in the Ctrl hydrogels (Fig. 2C, **bottom**).

MSCs in the Ab+ group exhibited a larger cell size ($11,000 \pm 600 \mu\text{m}^3$) when compared to untreated MSCs in Ncad ($7,600 \pm 500 \mu\text{m}^3$) and Ctrl ($8,000 \pm 300 \mu\text{m}^3$) hydrogels (Fig. 3B). Interestingly, similar to the membrane β cat findings, Ab+ MSCs adopted β cat distributions similar to untreated MSCs in Ctrl hydrogels (Fig. 3C). Roundness values between Ab+ and Ctrl groups were not significantly different and MSCs were significantly less round than untreated MSCs in Ncad hydrogels (Fig. 4A). Nuclear β cat of Ab+ MSCs was also significantly lower than MSCs in the Ncad group, and were indistinguishable from MSCs in the Ctrl group (Fig. 4B). Additionally, the nuclear β cat histogram curve of MSCs in the Ab+ group followed a similar trend to the curve from the Ctrl group (Fig. 4C), and the nuclear β cat versus cell roundness scatter plot showed Ab+ MSCs clustering in a similar manner to MSCs in the Ctrl hydrogels (Fig. 4D).

Cell-cell interactions enhance nuclear β cat localization

Since Ncad interactions result in the formation of adherens junctions between two cells and initiate downstream signaling,²⁷ we next sought to determine whether cell-cell interactions

also enhance nuclear β cat localization. Although the majority of cells photoencapsulated within hydrogels were distributed as single cells, a small percentage of cells were also encapsulated as two-cell clusters (Fig. 5A). As such, the same culture conditions were used to investigate the role of cell-cell interactions on nuclear β cat localization in two-cell clusters of MSCs photoencapsulated in either Ncad or Ctrl hydrogels in comparison to single cell outcomes (Fig. 5B). Representative actin and nuclei images of two-cell clusters in the Ncad and Ctrl conditions featured similar cellular and nuclear morphologies and were otherwise indistinguishable from one another (Fig. 5C), following the same trend observed for single cells in those same groups (Fig. 3B). However, unlike single cells in Ncad and Ctrl groups, where a large decrease in nuclear β cat was observed in Ctrl hydrogels (Fig. 3C), no differences in β cat signal for the same two-cell clusters were observed, regardless of peptide condition (Fig. 5D).

Nuclear β cat was determined for single and two-cell clusters in Ncad and Ctrl hydrogels (Fig. 5E). There was a 20% increase in nuclear β cat between single cells and two-cell clusters in the Ncad group. Similarly, there was an almost a two-fold increase in nuclear β cat in two-cell clusters in Ctrl hydrogels, relative to single cells in this gel. Additionally, no statistical differences were observed between two-cell clusters cultured in Ncad and Ctrl hydrogels. Nuclear β cat histograms of two-cell clusters in the Ncad and Ctrl groups show similar nuclear β cat distributions (Fig. 5F). Histograms of single versus two-cell clusters show similar trends for the Ncad hydrogels (Fig. 5G); however, more pronounced differences in nuclear β cat distributions between single cells and two-cell clusters were observed in Ctrl hydrogels (Fig. 5H).

Discussion

Hydrogels engineered to contain cues that mimic developmental and native microenvironments can significantly enhance chondrogenesis.⁴ However, the molecular mechanisms conducive to chondrogenesis within hydrogels remain largely unexplored, beyond population analyses of gene expression and resulting matrix formation. This is particularly important as MSC heterogeneity has been implicated in such systems and a better understanding of this phenomenon may lead to further improvements in MSC-based approaches for cartilage repair. To address these limitations, we utilized confocal imaging to investigate single-cell behavior within hydrogels.

Our laboratory recently engineered HA hydrogels that inherently interact with receptors, including CD44 and CD168, through the native biopolymer backbone to stimulate chondrogenesis (e.g., upregulation of chondrogenic genes including type 2 collagen and aggrecan),^{4,26,31} as well as to mimic cell-cell interactions through inclusion of an Ncad peptide that presents signals found during embryonic limb bud development.¹⁵ In addition to promoting cellular condensation,^{2,11} Ncad recruits β cat to the membrane and helps prevent it from being degraded by the GSK3 complex in the cytosol, which increases available β cat and allows for its translocation to the nucleus.¹⁸ Several studies also cite the importance of cell shape during stem cell differentiation.^{9,13,20} For instance, Chen and coworkers showed that in the presence of TGF- β 3, MSCs preferentially differentiate towards a chondrogenic lineage when confined to a round morphology.⁹

With these features in mind, single cell analyses were performed after 3 days in culture, since we previously showed that Ncad mimetic peptides induced increased expression of chondrogenic genes at this time point.⁴ Likewise, modifications to hydrogels are likely to influence cells at early times, prior to extensive tissue production that can lead to signaling from produced matrix molecules. The hydrogels were designed so that all features were similar, including hydrogel mechanics and peptide concentration, and the only difference between groups was the peptide sequence included. The non-active peptide was a scrambled sequence of the active domain, so that other features of the peptide such as charge were normalized across groups. With this approach, the groups that contained Ncad peptides led to a significant increase in cell roundness and β cat localization at both the membrane and nucleus. It is important to note that in this study only one concentration of active and control peptide was investigated; however, this concentration led to differences in cell response. Using the imaging technique presented, a more broad range of concentrations could be investigated to determine whether there are peptide concentrations that induce even greater signaling and chondrogenesis.

To assess the extent of heterogeneity in stem cell responses, nuclear β cat histograms were plotted. Although histogram peaks for nuclear β cat values in Ncad hydrogels were higher than in Ctrl hydrogels, there was a high degree of heterogeneity under all conditions. For instance, some cells in the Ctrl condition had nuclear β cat values representative of MSCs in Ncad hydrogels and vice versa. To identify potential relationships between cell morphology and β cat nuclear localization, nuclear β cat versus roundness scatter plots were created, showing a positive correlation between nuclear β cat localization and cell roundness.

To show that Ncad mimetic peptide-induced roundness and β cat localization at the membrane and in the nucleus was due to Ncad peptide interactions with Ncad transmembrane proteins, two experiments were performed. First, Ncad transmembrane proteins were blocked by treating MSCs with an anti-GC-4 Ncad antibody prior to photoencapsulation in Ncad hydrogels. The GC4 clone was chosen due to its selective binding affinity to the N-terminal half of the extracellular domain of human Ncad.⁴ Thus, the goal of the antibody was to block cellular interactions with the Ncad peptides, resulting in MSC behavior of membrane/nuclear β cat and roundness similar to that of MSCs in Ctrl hydrogels without Ncad peptides. At a concentration that did not compromise cell viability, antibody-treated MSCs exhibited a significant drop in roundness and membrane/nuclear β cat localization, indicating that Ncad peptides interact with Ncad transmembrane proteins. Furthermore, a nuclear β cat versus roundness scatter plot showed a clear separation between the two conditions and a positive correlation between these metrics, analogous to trends observed between untreated MSCs in Ctrl and Ncad hydrogels.

As another indication of the peptide induction of cell-cell interactions, we separated out findings of single cells with two-cell clusters observed within the hydrogels. In the images analyzed at 3 days, about 15% of cells formed clusters (data not shown). This low percentage of clusters is similar to what we observe immediately after encapsulation; thus, we believe this to be due to the encapsulation technique rather than proliferation. Two-cell clusters in both control and Ncad hydrogels reached the same levels of β cat nuclear localization. The nuclear β cat achieved was slightly higher than levels attained by single

MSCs in Ncad hydrogels, suggesting that there is room for improvement in the signaling from the peptide modified hydrogels to reach values of cell-cell contact. In contrast, comparisons of single cells and two-cell clusters in the Ctrl hydrogels (without the Ncad peptides) showed a much larger increase in nuclear β cat, indicating that cell-cell contacts alone significantly enhanced β cat nuclear localization when the Ncad signal was not included. Thus, the peptide modification is a significant improvement when single cells are encapsulated and in combination with the benefits of using hydrogels in cartilage repair represents and advance for the field.

To summarize, we utilized confocal imaging to probe single cell responses to Ncad mimetic peptides of photoencapsulated MSCs. We found that the presence of Ncad mimetic peptides increased β cat accumulation at the cell membrane and within nucleus as well as MSC roundness. Using histograms, we were able to identify the extent of heterogeneity in these hydrogels, highlighting the potential use of this method as a screening tool to identify conditions that reduce heterogeneity. We also found that situations of multiple cells (two-cell clusters) rather than single cells resulted in enhanced nuclear β cat in both Ncad and Ctrl hydrogels, with a much more pronounced effect in the Ctrl group. Varying cell cluster size and Ncad peptide concentrations may further enhance nuclear β cat localization and downstream Ncad-mediated cartilage production. In conclusion, the methods presented show promise in identifying candidate biomaterials to further the use of stem cells in cell-based chondrocyte repair strategies.

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Figure 1.

Experimental design and viability. **(A)** MeHA macromers were functionalized with either Ncad mimetic peptides (Ncad, red) or non-active scrambled control peptides (Ctrl, blue) and used to encapsulate MSCs in the presence of initiator and light. **(B)** Schematic of blocking Ncad transmembrane proteins prior to the encapsulation of MSCs within Ncad modified hydrogels by exposure of MSCs to Ncad antibodies (Ab+, green). **(C)** Live/dead imaging (live cells green, dead cells red) of MSC-laden hydrogels at 3 days. MSC viability was high in all groups (>95%). (Scale bar, C = 200 μm).

Figure 2.

Membrane β cat determination and staining of single MSCs in Ncad and Ctrl hydrogels. (A) Overview of semi-quantitative assessment of membrane β cat. 3-dimensional reconstruction of single MSCs stained for actin (red) and β cat (green) were used to determine the centroid of the cell. A two-pixel thick rectangle was then used to calculate a horizontal pixel intensity profile that spanned the width of the cell. (B) Representative cross-section images of single MSCs in Ncad (red rectangle, left), Ctrl (blue rectangle, middle), and Ab+ MSCs in Ncad (green rectangle, right) hydrogels stained for β cat at day 3. Normalized β cat intensity profiles (grey lines for individual cells, thick colored line for average) for MSCs in (C) Ncad (top, red) and Ctrl (bottom, blue) hydrogels, as well as (D) when antibodies were used to block Ncad interactions prior to MSC encapsulation (Ab+, green). (Scale bar, B = 200 μ m).

Figure 3.

Nuclear βcat quantification workflow and images of single MSCs in Ncad and Ctrl hydrogels. **(A)** To assess how single MSCs interact with Ncad mimetic peptides, actin (red), nucleus (blue), and βcat (green) channels were separated, and Otsu's intensity thresholding method was used to generate 3-dimensional masks (V_{cell} , V_{nuc} , V_{cyt}) for each channel. Next, βcat image stacks were superimposed with V_{nuc} and V_{cyt} to obtain βcat intensity in the cytosolic (βcat_{cyt}) and nuclear (βcat_{nuc}) domains. V_{cell} was used to determine cell roundness (1 = perfect sphere) and nuclear βcat was calculated as the ratio (nuclear βcat) between βcat_{nuc} and βcat_{cyt} normalized to V_{nuc} and V_{cyt} , respectively. Representative **(B)** maximum and **(C)** average projections of single MSCs photoencapsulated in MeHA hydrogels (Ncad: red, left; Ctrl: blue, middle; Ab+: green, right) after 3 days and stained for (B) actin (red), nucleus (blue), and (C) βcat (green). (Scale bars, B,C = 25 μm).

Figure 4.

Roundness and β cat signaling of single MSCs in Ncad and Ctrl hydrogels after 3 days of culture. Outcomes of average (A) cell roundness and (B) β cat Ratio between groups (Ncad: red; Ctrl: blue; Ab+: green). The groups containing the Ncad peptide had statistically greater cell roundness and nuclear β cat compared to Ctrl hydrogels and Ab+ MSCs in Ncad hydrogels; no statistical differences were observed between the Ctrl and Ab+ groups. To better illustrate heterogeneity in groups, nuclear β cat is also plotted as (C) histograms and (D) scatter plots in correlation with cell roundness. * indicates statistical significance ($p < 0.01$) versus the Ncad group.

Figure 5.

Comparison of single- and two-cell clusters. (A) Representative maximum projection (actin: red, nuclei: blue) of single- and two-cell clusters within a hydrogel. (B) Schematic of multiple cells interacting with each other as well as with hydrogels containing either Ncad or Ctrl peptides. (C) Maximum projections derived from 3-dimensional image stacks of two-cell clusters in Ncad (left) and Ctrl (right) hydrogels stained for actin (red) and nuclei (blue). (D) Average projections of the same clusters immunostained for β cat (green). (E) Nuclear β cat of single cells and two-cell clusters in the Ncad and Ctrl hydrogels. There is no significant difference in nuclear β cat between two-cell clusters in the Ncad and Ctrl hydrogels; however, there is a slight increase in nuclear β cat between single and two-cell clusters in the Ncad hydrogels and an almost two-fold increase in nuclear β cat in the clusters when compared to single cells in the Ctrl hydrogels. Histograms of (F) two-cell clusters in Ncad (red) and Ctrl (blue) hydrogels and two-cell clusters (solid lines) versus single MSCs (dashed lines) in (G) Ncad hydrogels and in (H) Ctrl hydrogels. (Scale bars, A,C,D = 25 μ m). * indicates statistical significance ($p < 0.01$) versus single cells group.