Human amniotic membrane modulates Wnt/β-catenin and NF-κβ signaling pathways in articular chondrocytes in vitro

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Abstract

Objective: Inflammation, catabolism, and hypertrophy in chondrocytes play a central role in osteoarthritis (OA). The Wnt/β-catenin and NF-κβ pathways contribute to these degradative processes. This in vitro study evaluates the inhibitory effect of a novel therapeutic, micronized dehydrated human amnion/chorion membrane (μHACM), as a potential treatment to offset elevated Wnt/β-catenin and NF-κβ signaling.

Design: Three-dimensional human articular chondrocyte pellets were stimulated with an inflammatory cocktail to induce a degenerative phenotype. Treatments included varying doses of μHACM. Protein and gene expression were analyzed using qRT-PCR, immunoblotting, and immunofluorescence to assess changes in the major constituents of Wnt/β-catenin and NF-κβ signaling. Regulation of catabolic activity was evaluated using enzymatic assays that detect MMP-13 and aggrecanase-mediated degradation products in conditioned media.

Results: Confirmation of the model was established through the expression of specific markers and extracellular matrix genes, verifying a chondrogenic phenotype was maintained. Inflammatory stimulation elicited a change in the chondrocyte proteome and secretome, elevating Wnt/β-catenin and NF-κβ expression/nuclear localization of the Wnt signaling axis and inhibiting IKKα, phospho-IκBα, and phospho-p65 in the NF-κβ signaling cascade. Additionally, μHACM altered expression of direct downstream targets, namely MCP1, MMP3, MMP13, ADAMTS4, ADAMTS5, RUNX2 and COL10A1. Moreover, μHACM reduced MMP-13 and aggrecanase-mediated substrate degradation.

Conclusion: μHACM ameliorated the effects of inflammatory-induced degeneration in chondrocytes through Wnt/β-catenin and NF-κβ inhibition, subsequently downregulating key inflammatory, hypertrophic and catabolic mediators in vitro.

1. Introduction

Two fundamental molecular pathways implicated in osteoarthritis (OA)-driven articular cartilage (AC) degeneration are Wnt/β-catenin [1] and NF-κβ [2]. These pathways are thought to be overactivated in OA; therefore, regulation of both Wnt and NF-κβ is emerging as a potential therapeutic target. The current non-surgical treatment paradigm for OA focuses on acute pain relief and reducing inflammation, failing to address degenerative structural damage to the AC. Micronized dehydrated human amnion/chorion membrane (μHACM; MiMedx Group, Inc.), an injectable advanced biologic, is explored as an alternative solution to the shortcomings of traditional treatments. Previously published animal studies [3] and early outcomes in clinical studies [4–6] have demonstrated success using μHACM for OA, notably resulting in symptomatic improvements and pain reduction; however, the molecular mechanisms by which this is accomplished is unknown. μHACM is a PURION® processed allograft, which retains regulatory proteins inherent to native amniotic tissues. These bioactive proteins elicit responses from recipient cells to regulate functions, such as proliferation, fibrosis, chemotaxis and biosynthesis [7–12]. Therefore, this research aims to investigate the role of μHACM in modulating pathways implicated in OA disease progression, in vitro.

Wnt/β-catenin signaling, herein referred to as Wnt, governs the transcription of downstream effector genes through stabilization of nuclear transcription factor, β-catenin. In the absence of Wnt activation, β-catenin is degraded by a multi-protein ‘destruction complex.’ However,
upon interaction of Wnt ligands to Frizzled and LRP-5/6 co-receptors, the cytoplasmic protein disheveled (Dvl) is recruited to the membrane [13]. Activated Dvl sequesters GSK-3β and Axin, two core components of the ‘destruction complex.’ Subsequent phosphorylation of GSK-3β renders the kinase inactive [14], preventing assembly of the complex and ultimately stabilizing β-catenin. β-catenin translocates into the nucleus where it binds to coactivators T-cell factor/lymphoid enhancer factor (TCF/LEF) to induce transcription of genes that augment proliferation, osteogenic differentiation, and matrix mineralization of chondrocytes [15].

NF-κβ represents another family of inducible transcription factors, which regulates genes that potentiate inflammation and matrix turnover within the AC [2]. The NF-κβ pathway responds to diverse stimuli, most notably TNF-α, IL-6, and IL-1β, inflammatory cytokines upregulated in human OA cartilage [16]. In response to stimuli, the IKK complex, consisting of IKKa/IKKβ subunits and IKKγ regulatory subunit, is activated. IKK phosphorylates IκBα, an inhibitor of NF-κβ, triggering ubiquitin-dependent IκBα degradation and consequent release and phosphorylation of NF-κβ factors, most commonly p65/p50. This heterodimer localizes to the nucleus where it facilitates transcription of genes that stimulate inflammatory and catabolic responses.

In this study, an in vitro system was developed to induce degenerative changes in a three-dimensional chondrocyte pellet culture. The effect of μHACM was evaluated on the ensuing pathways and their downstream regulation of inflammation, proteolysis and hypertrophy. Specifically, Wnt and NF-κβ were investigated for their contribution to degenerative processes. It is hypothesized that μHACM inhibits these pathways to offset the effects of the inflammatory stimuli.

2. Methods

2.1. Chondrocyte culture

Normal human articular chondrocytes (NHACs) isolated from knee hyaline cartilage were cultured according to manufacturer’s instructions (Lonza). Culture reagents are described in Table S1. Following expansion up to passage four and <15 population doublings, NHACs were plated in either a monolayer or pellet culture for determining the optimal chondrogenic system. For monolayer cultures, cells were plated at 3200 cells/well in 96-well flat-bottom plates (Corning). For high-density pellet cultures, cells were plated at 100,000 cells/well in 96-well V-bottom plates coated with 5% Pluronic-F127 (Sigma-Aldrich) to prevent cellular attachment to the surface. Cells were centrifuged at 210 RCF for 3 m to form 3D pellets. To induce chondrogenic redifferentiation, NHACs were cultured in complete Chondrocyte Differentiation Medium (CDM, Lonza) for seven days. 10 ng/mL TGF-β3 (Peprotech) was added fresh for each medium change every other day. NHACs were grown in a humidified incubator at 37 °C containing 5% CO2. Subsequent μHACM-treatment experiments used pellet cultures.

2.2. In vitro cytokine stimulation model

Differentiated NHAC pellets were gently washed in basal CDM™ (bCDM™, Lonza) and stimulated with 10 ng/mL TNF-α (Promega) and 10 ng/mL Oncostatin M (OSM; R&D Systems) for 24 h. bCDM™-only was added to unstimulated control pellets.

2.3. Processing micronized dehydrated human amnion/chorion membrane (μHACM)

μHACM (MiMedx Inc.) is an injectable dehydrated human allograft, comprised of micronized and sieved laminated amnion/chorion membranes derived from placental tissue. See Supplementary Materials and Methods.

2.4. μHACM extract preparation and treatment

Extracts of soluble factors from μHACM were prepared as previously described [10]. Briefly, μHACM tissue was extracted at 10 mg/mL in bCDM™ for 24 h at 4 °C with gentle rotation. Previous studies have established a significant proportion of soluble growth factors and cytokines in μHACM elute under these conditions [8]. Extracts were diluted in bCDM™ to the desired testing concentrations (5.0, 2.5, or 0.2 mg/mL). Following 24 h TNF-α/OSM stimulation, triplicate NHAC cultures were treated with the desired μHACM concentration in the presence of TNF-α/OSM for 48 h. NHACs cultured in the presence of bCDM™ only and bCDM™-containing TNF-α/OSM were used for unstimulated and stimulated controls, respectively.

2.5. RNA isolation and qRT-PCR

RNA was extracted from NHACs and reverse transcribed to generate cDNA using the Cells-2-CT Kit (Thermo Fisher), according to manufacturer’s instructions. For gene expression quantitation, qRT-PCR was performed (6 replicates/sample) using the QuantStudio™ System (Thermo Fisher), TaqMan® Gene Expression Master Mix and Assays (Thermo Fisher) were used for amplification, according to manufacturer’s instructions (primers listed in Table S2). 18S ribosomal-rRNA was used as an endogenous reference gene control for normalization. The 2−ΔΔCt method [17] was used to quantify fold-change expression relative to unstimulated control.

2.6. Protein precipitation from conditioned medium for MMP-13 immunoblot

Conditioned media (CM) from unstimulated/stimulated/μHACM-treated NHACs were filtered using a 0.2 μm PVDF Membrane 96-Well Filter Plate (Corning). A modified protocol as previously described [18] was used for protein precipitation using trichloroacetic acid (TCA) and sodium deoxycholate (DOC) to obtain sufficient MMP-13 protein for immunoblotting. See Supplementary Materials and Methods.

2.7. Whole cell lysate isolation

NHACs were washed in ice-cold DPBS containing Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher). Protein was extracted by lysing cells using RIPA Buffer (Thermo Fisher) with added Halt™ Protease and Phosphatase Inhibitors. Pellets were agitated on a plate shaker at 4 °C for 30 m and sonicated at 20 kHz 3 times at 2 s/cycle. Lysates were centrifuged at 14,000 RCF for 15 m at 4 °C. Total protein content was determined using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher), according to manufacturer’s instructions. Whole cell lysates were used for total protein expression where determining subcellular localization was unnecessary.

2.8. Cytoplasmic and nuclear protein extraction

Fractional lysates were used for p65 immunoblotting to demonstrate site-specific location of its biochemical regulation which can occur in cytoplasmic or nuclear compartments. Subcellular fractions were extracted using NE-PER™ Reagents with added Halt™ Protease and Phosphatase inhibitors (Thermo Fisher), per manufacturer’s instructions. Samples were homogenized using a Dounce homogenizer. Total protein content of the fractionated extracts was determined as aforementioned.

2.9. SDS-PAGE/immunoblotting

Equal volume of precipitated samples or equal concentration of protein from lysates were reduced and denatured using Bolt™ LDS Sample Buffer and dithiothreitol (Thermo Fisher). Samples were loaded into Bolt™ Bis-Tris 4–12% polyacrylamide gels (Thermo Fisher) and resolved
by SDS-PAGE. Proteins were transferred to a nitrocellulose or PVDF membrane using the iBlot 2 System (Thermo Fisher), according to manufacturer’s instructions. The membranes were blocked in 5% milk/Tris-Buffered Saline, 0.1% Tween-20 (TBST). Immunoblotting was performed using antibodies in Table S2. ImageJ was used to quantitate band intensities which were normalized to GAPDH or Lamin B1 loading controls. For phosphorylation quantitation, phosphorylated protein and total protein were normalized to loading control, followed by calculating the normalized phosphorylated protein/total protein ratio.

2.10. MMP-13 enzymatic activity assay

To determine the proteolytic activity of secreted MMP-13 in the CM from stimulated/treated NHACs, Sensolyte® Plus 520 MMP-13 Assay (AnaSpec) was used according to manufacturer’s instructions. See Supplementary Materials and Methods.

2.11. Aggrecanase enzymatic activity assay

Aggrecanase activity in the CM from stimulated/treated NHACs was determined using the Sensitive Aggrecanase Activity Assay (BioTeZ) according to manufacturer’s instructions. See Supplementary Materials and Methods.

2.12. Immunofluorescence

Cryosectioning and immunostaining of treated NHAC pellets were performed according to standard procedures. See Supplementary Materials and Methods.

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0. Values are reported as mean ± 95% confidence interval (CI). One-way analysis of variance (ANOVA) was used to evaluate associations between multiple groups. Data sets that did not pass normality testing as evaluated by Gaussian residual distribution testing were transformed by Y = Log(Y). Post-hoc comparisons were made using Tukey multiple comparison procedure. For comparison of two groups, unpaired Student’s two-tailed t-test was used. A P-value of 0.05 or less (⁎P < 0.05, ⁎⁎P < 0.01, ⁎⁎⁎P < 0.001) was considered significant.

3. Results

3.1. Cytokine induction model of OA-like processes in an in vitro chondrocyte pellet system

Preliminary experiments were performed to verify the culture system preserved the expected chondrocyte phenotype. NHACs grown in a monolayer culture were compared to that of a three-dimensional high-density chondrocyte pellet culture by qRT-PCR of extracellular matrix genes, with ΔΔCt normalized to basal unstimulated group (herein referred to as Basal). Pellets exhibited the chondrocyte phenotype in contrast to the monolayer by expressing a 118-fold higher level of COL2A1 (95%CI = 150.90–213.40 ΔΔCt; P < 0.001) and a 4.4-fold higher level of ACAN (95%CI = 3.65–4.94 ΔΔCt; P < 0.001) [Fig. 1(a)]. Additionally, to confirm NHAC pellets did not undergo dedifferentiation or hypertrophy, we also examined COL1A1 and COL10A1 gene expression. The pellet culture expressed 27.8% less COL1A1 (95%CI = 0.63–0.82 ΔΔCt; P = 0.001) and 78.2% less COL10A1 (95%CI = 0.15–0.29 ΔΔCt; P < 0.001) compared to the monolayer culture. The COL2A1:COL1A1 ratio was 168.6-fold higher in pellets (95%CI = 218.20–284.8; P < 0.001) compared to monolayer. Imaging of a representative pellet displayed a cohesive, smooth surface with a uniform spherical shape and size between wells (diameter ~1 mm) [Fig. 1(b)]. Thus, subsequent experiments were conducted using the pellet culture system (herein referred to as NHACs) as it best exemplified the in vitro chondrocyte phenotype as suggested in the literature [19].

The addition of inflammatory stimuli was next evaluated to establish parameters that would produce an inflammatory, catabolic and hypertrophic phenotype in the NHAC pellets. Following TNF-α/OSM stimulation, COL2A1 was downregulated by 95.3% (95%CI = 0.03–0.06 ΔΔCt; P < 0.001) and ACAN was reduced by 86.6% (95%CI = 0.09–0.17 ΔΔCt; P < 0.001) compared to Basal [Fig. 1(c)]. TNF-α/OSM stimulation induced a 25.3-fold increase in MCP1 (95%CI = 17.38–33.51 ΔΔCt; P < 0.001 vs Basal). Moreover, TNF-α/OSM significantly elevated chondrocyte hypertrophy markers COL10A1 by 3.6-fold (95% CI = 1.88–3.70 ΔΔCt; P = 0.001) and RUNX2 by 2.6-fold (95% CI = 1.77–3.78 ΔΔCt; P = 0.01) compared to Basal. In regards to catabolic mediators, TNF-α/OSM significantly upregulated MMP3 by 445-fold (95%CI = 318.70–614.20 ΔΔCt; P < 0.001), MMP13 by 270-fold (95%CI = 447–1017 ΔΔCt; P < 0.001), ADAMTS4 by 30-fold (95% CI = 18.05–39.99 ΔΔCt; P < 0.001), and ADAMTS5 by 10.7-fold (95% CI = 8.70–22.26 ΔΔCt; P < 0.001). Together, these results established an appropriate model system, displaying a degenerative cartilage phenotype, in which to test the effects of µHACM.

3.2. µHACM inhibits Wnt/β-catenin signaling in stimulated chondrocytes

Modulation by µHACM of canonical Wnt signaling was evaluated in the TNF-α/OSM model. Gene expression for TCF/LEF transcription factors, specifically TCF4, TC7, and LEF1, was assessed using qRT-PCR. Compared to Basal, TNF-α/OSM stimulation induced a 62% increase in TCF4 (95%CI = 1.42–1.84 ΔΔCt; P = 0.001), a 173% increase in TC7 (95%CI = 1.56–3.51 ΔΔCt; P = 0.009), and a 104% increase in LEF1 (95%CI = 1.59–2.64 ΔΔCt; P = 0.025), indicating TNF-α/OSM induced downstream Wnt effector expression [Fig. 2(a)]. With µHACM treatment, a dose-dependent decrease in the expression of these Wnt-related transcription factors was observed. Compared to TNF-α/OSM group, 5.0 mg/mL µHACM reduced TCF4 by 71.3% (95%CI = 0.36–0.57 ΔΔCt; P < 0.001), TCF7 by 57.4% (95%CI = 0.77–1.39 ΔΔCt; P < 0.001), and LEF1 by 81.5% (95%CI = 0.22–0.56 ΔΔCt; P < 0.001). At 2.5 mg/mL, µHACM reduced TCF4 by 50.3% (95%CI = 0.65–0.98 ΔΔCt; P < 0.001), TCF7 by 41.9% (95%CI = 1.14–1.80 ΔΔCt; P = 0.049), and LEF1 by 72.1% (95%CI = 0.44–0.75 ΔΔCt; P < 0.001). At 0.2 mg/mL, expression levels were comparable to the TNF-α/OSM group.

Post-translational regulation of µHACM-mediated Wnt inhibition was further evaluated by immunoblotting for phospho-GSK-3β (total GSK-3β). Consistent with the downregulation of TCF/LEF transcription complex, µHACM induced a dose-dependent reduction in Ser9-phosphorylation of GSK-3β, with 5.0 and 2.5 mg/mL treatment reverting TNF-α/OSM-induced phosphorylation back to basal levels [Fig. 2(b)].

With phospho-dependent inactivation of GSK-3β, β-catenin levels were evaluated for accumulation within the cell. Total β-catenin protein levels were 63% higher following TNF-α/OSM stimulation, while 5.0 mg/mL µHACM reduced its expression to basal levels [Fig. 2(c)]. Following cytoplasmic accumulation of β-catenin, nuclear translocation of the protein would presumably ensue. Indeed, immunofluorescent imaging demonstrated both reduced nuclear translocation and total expression of β-catenin following 5.0 mg/mL treatment [Fig. 2(d)]. This inhibitory effect was absent when cells were treated with 0.2 mg/mL µHACM.

3.3. µHACM reduces expression of direct Wnt target genes associated with chondrocyte proliferation and hypertrophy

Downstream Wnt-regulated direct target genes associated with proliferation and hypertrophy in chondrocytes were evaluated. Compared to Basal, TNF-α/OSM increased Wnt genes that control proliferation, CYCLIN D1 (48.9% increase; 95%CI = 1.39–1.60 ΔΔCt; P = 0.011) and PCNA (46.4% increase; 95%CI = 1.05–1.89 ΔΔCt; P = 0.049) [Fig. 2(a)]. TNF-α/OSM did not have a significant effect on C-MYC. µHACM
Fig. 1. The establishment of normal and cytokine stimulated in vitro chondrocyte pellet model relevant to healthy and OA-like conditions. (a) Culture configuration-related changes in major chondrogenic markers, including COL2A1, COL1A1, ACAN, and COL10A1, as determined by qRT-PCR in NHAC monolayer vs 3D pellet culture following seven-day culture period in chondrogenic differentiation medium. Fold-change expression values were normalized to reference gene 18S and expressed relative to monolayer group (ΔΔCt). (b) Macroscopic image of representative NHAC pellet following 10 days in vitro. Top scale bar = 1 mm. Bottom image represents microscopically imaged NHAC pellet in brightfield using a 10 x objective. Bottom scale bar = 200 μm. (c) Fold-change in relative gene expression of chondrogenic, hypertrophic, inflammatory, and catabolic markers following 72h TNF-α/OSM (10 ng/mL) stimulation. Fold-change expression values were normalized to reference gene 18S and expressed relative to unstimulated group or Basal (ΔΔCt). Data represents mean ± 95% CI. Significant differences vs Basal are indicated as *P < 0.05, **P < 0.01, ***P < 0.001, as determined by unpaired student's two-tailed t-test, n = 3 biological replicates.
Fig. 2. μdHACM inhibits Wnt/β-catenin signaling pathway in TNF-α/OSM stimulated chondrocytes. (A) Relative gene expression of Wnt transcription factors, TCF4, TCF7, and LEF1, measured by qRT-PCR following 24-hr stimulation with TNF-α/OSM (10 ng/mL) and 48-hr treatment with μdHACM (5.0, 2.5, or 0.2 mg/mL). Data represents mean ± 95% CI. Significant differences vs TNF-α/OSM stimulated control are indicated as *P < 0.05, **P < 0.01, ***P < 0.001, as determined by one-way ANOVA and Tukey’s post-hoc test, n = 3 μdHACM donors. (B) Phosphorylation of GSK-3β at serine 9 and total GSK-3β protein levels measured by Western blot using whole cell lysates. Quantification of Western blot is shown. Phospho-GSK-3β and GSK-3β levels were first normalized to GAPDH, then the phosphorylated ratio was calculated from the normalized values and plotted as relative band intensity compared to Basal (denoted as (-) TNF-α/OSM); n = 2 μdHACM donors. (C) Protein expression of total β-catenin measured by Western blot. Quantification of Western blot is shown. β-catenin levels normalized to GAPDH levels and plotted as band intensity relative to Basal; n = 2 μdHACM donors. (D) Representative immunofluorescent images comparing Basal unstimulated, TNF-α/OSM (10 ng/mL) stimulated and μdHACM treated (5.0 and 0.2 mg/mL + TNF-α/OSM) groups to evaluate nuclear localization of β-catenin. Sections stained with β-catenin antibody (green) and DAPI (blue). The top row displays the pellet cross-section imaged using 10 × objective and the bottom row displays a representative (highlighted by white inset box in top image) magnified region (using 40 × objective). The white arrowheads in the bottom row images show examples of β-catenin localization patterns in each group. Images representative of four 10 μm-thickness cryosections/pellet (n = 3 μdHACM donors, 3 pellets/group). Data representative of at least 2 independent experiments.
2.5 mg/mL dose of μWISP1 by 29.1% (95%CI < P < 0.01, ***P < 0.001) compared to the TNF-α/OSM stimulated control are indicated as *P < 0.05, **P < 0.01, ***P < 0.001, as determined by one-way ANOVA and Tukey’s post-hoc test, n = 3). 

**P < 0.01, ***P < 0.001, and C-MYC by 38.9% (95%CI < 0.001), all by 50% compared to TNF-α/OSM treatment (95%CI = 1.38–2.64 ΔΔCt; P < 0.001), while no significant change was observed for HIF2A, as 2.5 mg/mL and 0.2 mg/mL also reduced HIF2A levels comparably (P = 0.024) [Fig. 5(a)]. **

TNF-α/OSM increased catabolic gene HIF2A by 2-fold compared to Basal (95%CI = 1.38–2.64 ΔΔCt; P < 0.001) and 57.6% with 2.5 mg/mL treatment (95%CI = 8.64–12.92 ΔΔCt; P = 0.002) [Fig. 5(a)]. Following 5.0 mg/mL μHACM treatment, HIF2A levels decreased by 37.7% (95%CI = 1.06–1.35 ΔΔCt; P = 0.008). The lower 2.5 mg/mL dose of μHACM treatment was also sufficient to reduce COL10A1 by 24.1% (95%CI = 0.46–1.89 ΔΔCt; P = 0.022). The inhibitory effect on hypertrophic genes was abolished at the lowest 0.2 mg/mL treatment dose.

3.4. μHACM inhibits NF-κB signaling in stimulated chondrocytes

NF-κB pathway regulation was evaluated as an additional mechanism of action for μHACM treatment effects in TNF-α/OSM-stimulated chondrocytes. As shown in the immunoblot [Fig. 4(a)], TNF-α/OSM stimulated group displayed elevated IKKβ levels. Increased IKKβ leads to increased kinases activity, specifically phosphorylation of substrates IκBα (or inhibitor of NF-κB) and p65. Indeed, TNF-α/OSM induced serine 32 phosphorylation of IκBα and serine 536 phosphorylation of p65 in the cytoplasm [Fig. 4(b)], which in turn increased phospho-p65 expression in the nucleus [Fig. 4(c)]. μHACM treatment dose-dependently reduced IKKβ protein levels and phosphorylation of IκBα. Further downstream, 5.0 mg/mL μHACM treatment reversed phospho-p65 (S536) induced by TNF-α/OSM in the cytoplasmic and nuclear extract, restoring phosphorylation modification to basal level.

μHACM modulates expression of downstream NF-κB target genes associated with inflammation and catabolic/anabolic regulation.

Downstream transcriptional regulation of direct NF-κB target genes, namely MCP1 [20], HIFIA [21], HIF2A [22], MMP3 [23], MMP13 [24], ADAMTS4 [25], and ADAMTS5 [26] in chondrocytes stimulated by TNF-α/OSM was evaluated by qRT-PCR. Relative to Basal, TNF-α/OSM stimulation significantly induced pro-inflammatory gene MCP1, which was dose-dependently reduced by 73.8% with 5.0 mg/mL μHACM treatment (95%CI = 3.64–9.68 ΔΔCt; P < 0.001) and 57.6% with 2.5 mg/mL treatment (95%CI = 8.64–12.92 ΔΔCt; P = 0.002) [Fig. 5(a)].

TNF-α/OSM increased catabolic gene HIF2A by 2-fold compared to Basal (95%CI = 1.38–2.64 ΔΔCt; P < 0.001) and 57.6% with 2.5 mg/mL treatment (95%CI = 8.64–12.92 ΔΔCt; P = 0.002). In contrast to HIF2A downregulation, 5.0 mg/mL μHACM treatment, HIF2A levels decreased by 37.7% (95%CI = 1.06–1.35 ΔΔCt; P = 0.008). It should be noted a dose-dependent response was not achieved for HIF2A, as 2.5 mg/mL and 0.2 mg/mL also reduced HIF2A levels comparably (P = 0.010 and 0.021, respectively). In contrast to HIF2A downregulation, 5.0 mg/mL μHACM treatment interestingly increased HIF1A by 2-fold (95%CI = 3.03–4.38 ΔΔCt; P < 0.001), a factor that counteracts the catabolic effects imparted by HIF2A.

Critical to driving ECM catabolic degradation, TNF-α/OSM strongly enhanced gene expression of the major MMPs and aggrecanases implicated in collagen and aggrecan degradation, specifically ADAMTS4, ADAMTS5, MMP3 and MMP13 [Fig. 5(c)]. 5.0 mg/mL μHACM treatment reduced ADAMTS4 (95%CI = 11.44–16.11 ΔΔCt; P = 0.008), ADAMTS5 (95%CI = 6.89–8.53 ΔΔCt; P < 0.001), and MMP3 (95%CI = 177.5–256.3 ΔΔCt; P < 0.001), all by 50% compared to TNF-α/OSM alone. Strikingly, MMP13 was reduced nearly 90% (95%
CI = 59.49–125.3 ΔΔCt; P < 0.001) with 5.0 mg/mL μdHACM treatment and even effectively reduced by 71% with 2.5 mg/mL μdHACM (95% CI = 161.8–263.2 ΔΔCt; P = 0.004).

3.5. μdHACM abolishes MMP-13 protein expression and activity in conditioned media

Due to the potent transcriptional inhibition of MMP-13 previously observed, levels of MMP-13 protein contained in CM as well as its enzymatic activity were further characterized. The levels of secreted MMP-13 under basal conditions were undetectable [Fig. 6(a)]. TNF-α/OSM stimulation induced MMP-13 protein expression. Two protein bands were detected: a prominent, higher MW band approximately 50-kDa and a faint, lower MW band approximately 29-kDa. The higher MW band likely represents active or intermediate-forms of MMP-13 and the lower MW band likely represents the catalytic domain produced following MMP-13 activation/autoproteolysis. Consistent with gene regulation, μdHACM treatment resulted in a decline in MMP-13 protein, where 5.0 mg/mL virtually abolished its expression. 2.5 mg/mL treatment produced a faint band and the inhibitory effect was negligible at 0.2 mg/mL.

μdHACM-mediated inhibition of MMP-13 was further characterized by enzymatic activity measurements. MMP-13 in CM was first immunoprecipitated to isolate MMP-13 and exclude other proteases. Whereas vehicle control (bCDM) displayed no MMP-13 activity, the positive control (rhMMP-13 + APMA) generated a robust signal (95% CI = 321.7–403.5 RFU/min; P < 0.001 vs vehicle) [Fig. 6(b)]. The test article control (5.0 mg/mL μdHACM + APMA in the absence of cells) did not result in any appreciable MMP-13 activity (95% CI = 0–11.09 RFU/min) and was not significantly different from vehicle (P > 0.999). NHACs stimulated with TNF-α/OSM secreted active MMP-13, as indicated by the increase in activity (95% CI = 92.33–137.7 RFU/min; P < 0.001 vs Basal). Consistent with the strong inhibitory effect observed in protein expression, 5.0 mg/mL μdHACM treatment was also most effective at suppressing MMP-13 activity to undetectable levels (P < 0.001 vs TNF-α/OSM group).

3.6. μdHACM attenuates ADAMTS-mediated aggregan degradation

CM from TNF-α/OSM-stimulated NHAC pellets treated with μdHACM
(5.0 or 0.1 mg/mL) was evaluated for aggrecan degradative activity by quantifying proteolyzed fragments (ARGSVIL-peptide) formed by the presence of aggrecanases. Basal-CM incubated with aggrecan-IGD for 15 min expectedly produced only minimal amounts of ARGSVIL-peptide fragments (mean $\bar{x} = 0.27$ nM; 95%CI = 0.11–0.48) [Fig. 6(c)]. In contrast, TNF-α/OSM-CM generated a 9-fold higher spike in ARGSVIL-peptide production (mean $\bar{x} = 2.41$ nM; 95%CI = 2.19–2.63; $P < 0.001$). Accordingly, specific aggrecanase activity (nM ARGSVIL/min·pM ADAMTS-4) increased from 0.15 in Basal-CM (95%CI = 0.05–0.26) to 2.54 nM ARGSSVL/min·pM ADAMTS-4 in TNF-α/OSM-CM (95%CI = 2.14–2.94; $P < 0.001$). Following 5.0 mg/mL μdHACM treatment, ARGSVIL-peptide concentration was diminished by 43% (mean $\bar{x} = 1.3$ nM; 95% CI = 0.86–1.85; $P = 0.005$) compared to the TNF-α/OSM group. This corresponded to the calculated reduction observed in aggrecanase activity (mean = 1.07; 95%CI = 0.56–1.59; $P = 0.005$). This inhibitory effect on aggrecanase activity was not observed with 0.1 mg/mL μdHACM treatment ($P = 0.994$).

Model for μdHACM inhibition of canonical Wnt/β-catenin and NF-κβ signaling pathways. Fig. 7 summarizes the proposed mechanism of action by which μdHACM regulates Wnt and NF-κβ pathways in an in vitro inflammatory system. For the Wnt pathway, μdHACM prevents phosphorylation of GSK-3β. Non-phosphorylated GSK-3β allows for targeting β-catenin for degradation, which prevents its accumulation and nuclear translocation, ultimately suppressing Wnt target genes. Additionally, μdHACM inhibits NF-κβ by downregulating IKKβ, which results in reduced phosphorylation of IκBα and p65. When Ser32-IκBα is in a non-phosphorylated state, IκBα becomes readily available to inhibit p65 nuclear translocation, thus preventing transcription of NF-κβ target genes.
4. Discussion

Chondrocytes, the only cell type comprising the AC, are responsible for matrix synthesis and turnover. Any disruption to this delicate balance, whether that be trauma, altered biomechanical loads or inflammatory signaling cues, may lead to matrix degeneration. This study utilized a method well-characterized in vitro [27–29] for inducing a degenerative phenotype in chondrocyte pellets to test the potential benefits of a novel treatment, μdHACM. The results suggest μdHACM indeed functions through modulation of pathways contributing to the disease state.

The observed reduction in COL2A1 and ACAN gene levels in NHAC pellets stimulated with TNF-α/OSM (10 ng/mL) and subsequently treated in the presence of TNF-α/OSM with μdHACM (5.0, 2.5 or 0.2 mg/mL). First eight lanes of immunoblot are precipitated samples. Last two lanes represent “Input”, where CM was directly added to the wells without precipitation. (-) TNF-α/OSM represents Basal unstimulated control. Graph displays quantification of band intensity of the higher MW band (~50-kDa) relative to Basal expression; n = 2 μdHACM donors.

(b) MMP-13 enzymatic activity calculated as mean RFU/min ±95% CI. First three bars represent cell-free assay controls following APMA activation, where Vehicle = bCDM Media; Test Article = 5.0 mg/mL μdHACM extract (n = 3 μdHACM donors), and rhMMP-13 = 200 ng/mL catalytic domain rhMMP-13. Last five bars represent activity in non-precipitated CM samples. (c) Quantification of ARGSVIL-peptide concentration and aggrecanase activity in CM obtained following TNF-α/OSM (10 ng/mL) stimulation and μdHACM treatment (5.0 or 0.1 mg/mL). Samples incubated with 1 μM rhAggrecan-IGD for proteolysis reaction followed by fragmented ARGSVIL-peptide ELISA quantification. Data represents mean ± 95% CI. Significant differences vs TNF-α/OSM stimulated control indicated as *P < 0.05, **P < 0.01, ***P < 0.001 as determined by one-way ANOVA and Tukey’s post-hoc test, n = 3 μdHACM donors. Data representative of at least 2 independent experiments.

Fig. 6. Anti-catabolic regulation of MMP-13 protein expression and enzymatic activity following μdHACM treatment. (a) Representative immunoblot of secreted MMP-13 protein levels contained in the conditioned media (CM) of NHAC pellets stimulated with TNF-α/OSM (10 ng/mL) and subsequently treated in the presence of TNF-α/OSM with μdHACM (5.0, 2.5 or 0.2 mg/mL). First eight lanes of immunoblot are precipitated samples. Last two lanes represent “Input”, where CM was directly added to the wells without precipitation. (-) TNF-α/OSM represents Basal unstimulated control. Graph displays quantification of band intensity of the higher MW band (~50-kDa) relative to Basal expression; n = 2 μdHACM donors.

(b) MMP-13 enzymatic activity calculated as mean RFU/min ±95% CI. First three bars represent cell-free assay controls following APMA activation, where Vehicle = bCDM Media; Test Article = 5.0 mg/mL μdHACM extract (n = 3 μdHACM donors), and rhMMP-13 = 200 ng/mL catalytic domain rhMMP-13. Last five bars represent activity in non-precipitated CM samples. (c) Quantification of ARGSVIL-peptide concentration and aggrecanase activity in CM obtained following TNF-α/OSM (10 ng/mL) stimulation and μdHACM treatment (5.0 or 0.1 mg/mL). Samples incubated with 1 μM rhAggrecan-IGD for proteolysis reaction followed by fragmented ARGSVIL-peptide ELISA quantification. Data represents mean ± 95% CI. Significant differences vs TNF-α/OSM stimulated control indicated as *P < 0.05, **P < 0.01, ***P < 0.001 as determined by one-way ANOVA and Tukey’s post-hoc test, n = 3 μdHACM donors. Data representative of at least 2 independent experiments.
chondrocyte maturation and hypertrophy [39,40], since overexpression of RUNX2 in chondrocytes induces hypertrophy and endochondral ossification [41]. COL10A1, a hypertrophic chondrocyte-specific marker, is also a direct transcriptional target of RUNX2 [42].

The mechanism by which μdHACM regulates Wnt signaling is hypothesized to be direct delivery of Wnt inhibitors, inherent to amniotic tissue. For example, significant levels of DKK1 and IGFBP-3 are known to be present in amniotic tissue and have been detected in μdHACM [9,43]. DKK1 antagonizes Wnt ligand binding preventing signal transduction [44] by forming a complex with LRP5/6 and Kremen, followed by endocytosis of this complex and removal of LRP5/6 from the cell surface. IGFBP-3 inhibition of the Wnt pathway is dependent upon the presence of CD44, a receptor protein known to modulate Wnt signaling. Upon cytoplasmic entry, IGFBP-3 binds the Wnt complex, interacting specifically with GSK-3β. As a consequence, the β-catenin destruction complex dissociates from LRP6 and GSK-3β is activated by dephosphorylation, becoming free to target β-catenin [45]. Together, this suggests a mechanism by which μdHACM neutralizes the effects of the inflammatory stimuli through Wnt-inhibition of downstream targets responsible for hypertrophy and proteoglycan loss in diseased chondrocytes.

NF-κβ signaling was also upregulated in TNF-α/OSM-stimulated chondrocytes. μdHACM treatment resulted in decreased IKKβ and phosphorylation of IκBα, essentially functioning to stabilize IκBα binding to NF-κβ transcription factors. Accordingly, a decrease in phosphorylated-p65 was observed in both cytoplasmic and nuclear fractions. Downstream, the expression of direct NF-κβ target genes (MCP1, HIF1A, HIF2A, ADAMTS4, ADAMTS5, MMP3 and MMP13) were downregulated. MCP1 is a potent inflammatory factor known to initiate chondrocyte degradation and elicit further damage through a positive feedback mechanism [46]. HIF2A directly induces the expression of catabolic factors and regulates autophagy in chondrocytes to mediate apoptosis [47]. ADAMTS-4 and -5 are proteases that are responsible for aggrecan degradation, a primary matrix component of AC. A degradation assay translated the observed downregulation of aggrecanase genes into a post-translational outcome, where μdHACM suppressed aggrecanase-specific proteolysis. MMP-13 is specifically expressed in the cartilage of human OA patients and possesses the highest activity against type II collagen, the other major structural component of AC [48]. Similar to the aggrecanase activity findings, a functional proteolysis assay confirmed MMP-13 downregulation corresponded to reduced MMP-13-mediated degradation. Overall, μdHACM suppressed NF-κβ signaling to preserve the primary constituents of the chondrocyte matrix.

The proposed mechanism by which μdHACM inhibits NF-κβ signaling is similarly through antagonist delivery. Known inhibitors of the NF-κβ signaling pathway, including IGFBP-3 and -5, have been previously reported to be detected within μdHACM [9]. IGFBP-3 downregulates TNF-α-induced expression of IκBα and p65 through IGFBP-3R-mediated activation of caspases [49]. IGFBP-5 functions to inhibit TNF-R1 to block signal transduction [50]. Collectively, the presence of these factors suggests a mechanism by which μdHACM is able to oppose the effects of an inflammatory environment and protect the matrix integrity through inhibition of NF-κβ signaling.

While several pathways are responsible for degeneration in chondrocytes, regulation of Wnt and NF-κβ signaling demonstrates contributing mechanisms by which μdHACM modulates chondrocyte activity.
Despite little evidence to support matrix regeneration, these results indicate the potential for imparting a chondroprotective effect and attenuating degeneration in vitro. This study substantiates previous clinical [4-6] and pre-clinical evidence suggesting dHACM treatment improves the outcomes associated with OA. Specifically, dHACM attenuated the onset of surgically-induced OA in a medial meniscal transection (MMT) rat model [3]. dHACM administrated 24 h post-MMT remained in the synovium for 21 days and prevented cartilage degradation in the form of higher proteoglycan levels, fewer erosions, and no lesions, and now in vitro evidence make dHACM a candidate to become an effective therapeutic against arthritic degradation. The impact of dHACM on the synovium and interplay between all tissues will be necessary studies to correlate in vitro data to clinical relevance.

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Conception and Design: CC, MM, TJK; Acquisition, analysis and interpretation of data: CC; Drafting of manuscript: CC, MM; revision and final approval of manuscript: CC, MM, TJK.

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Declaration of competing interest
MiMedx Group, Inc. salary: Connie Chung, Michelle Massese, Thomas J Koob. MiMedx Group, Inc. equity: Michelle Massese, Thomas J Koob.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jocart.2021.100211.

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