Published in final edited form as:

ACS Biomater Sci Eng. 2019 April 8; 5(4): 1661–1667. doi:10.1021/acsbiomaterials.9b00070.

Molecular Detection and Assessment of Intervertebral Disc Degeneration via a Collagen Hybridizing Peptide

Li Xiao^{†,‡,§}, Rahul Majumdar^{†,‡,§}, Jun Dai^{†,‡}, Yang Li[§], Lin Xie^{†,||}, Francis H. Shen[†], Li Jin[†], Xudong Li^{*,†,⊥}

[†]Department of Orthopaedic Surgery, University of Virginia, 135 Hospital Drive, Cobb Hall, Charlottesville, Virginia 22908, United States

[‡]Department of Orthopaedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Qiaokou District, Wuhan 430030, P.R. China

[§]Department of Biomedical Engineering, University of Utah, 201 Presidents Circle, Salt Lake City, Utah 84112, United States

^{||}Department of Orthopaedics, Huashan Hospital, Fudan University, 12 Wulumuqi Middle Road, Jinan District, Shanghai 200040, P.R. China

[⊥]Department of Biomedical Engineering, University of Virginia, 415 Lane Road, Charlottesville, Virginia 22904, United States

Abstract

During aging, wear, and tear of intervertebral discs, human discs undergo a series of morphological and biochemical changes. Degradation of extracellular matrix proteins, e.g., collagen, arises as an important contributor and accelerator in this process. Existing methods to detect collagen degradation at the tissue level include histology and immunohistochemistry. Unfortunately, most of these methods only depict overall collagen content without the ability to specifically discern degraded collagen and to assess the severity of degeneration. To fill this technological gap, we developed a robust and simple approach to detect and assess early disc degeneration with a collagen hybridizing peptide (CHP) that hybridizes with the flawed triple

*Corresponding Author x12n@virginia.edu. Phone: 434-982-4135. Fax: 434-924-1691.

#Author Contributions

L.X. and R.M. equally contributed to the work. L.X., L.J., and X.L. designed the experiments. L.X. and R.M. acquired and analyzed the data. L.X., R.M., Y.L., F.H.S., and L.J. interpreted the data. L.X. and R.M. wrote the manuscript. L.J., L.X., and X.L. revised the manuscript critically. All authors have read and approved the final submitted manuscript.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsbiomaterials.9b00070](https://doi.org/10.1021/acsbiomaterials.9b00070). Detailed explanations on innovation and significance, distinct detection mechanism, and potential opportunity for *in vivo* imaging; characterization data of F-CHP; *ex vivo* dissection of mouse intervertebral disc; protocols of *in vitro* culture and F-CHP staining of mouse IVD; protocol of fluorescence image quantification; exploration on autofluorescence of mouse disc tissue (Figure S1) and weak staining in mouse disc NP (Figure S2); blocking study of CHP in mouse disc under inflammatory conditions (Figure S3); Alcian blue/picrosirius red staining and histological scoring protocols; correlation between F-CHP fluorescence intensity and histological scoring; histological analysis of paraffin embedded mouse disc samples (Figure S4); side by side comparison of immunohistochemistry (type II collagen) and CHP in mouse disc tissue (Figure S5); analysis of human IVD samples; and statistical analysis (PDF)

Notes

The authors declare the following competing financial interest(s): Y.L. is co-founder and shareholder of 3Helix Inc, which commercializes the collagen hybridizing peptides.

helix structure in degraded collagen. Intriguingly, the CHP signal in mouse lumbar discs exhibited a linear incremental pattern with age. This finding was corroborated with histological analysis based on established methods. When comparing this analysis, a positive linear correlation was found between CHP fluorescence intensity and the histological score with a regression value of $r^2 = 0.9478$. In degenerative mouse discs elicited by pro-inflammatory stimuli (IL-1 β and LPS) *ex vivo*, the newly developed approach empowered prediction of the severity of disc degeneration. We further demonstrated higher CHP signals in a degenerative human disc tissue when compared to a normal sample. These findings also resonated with histological analysis. This approach lays a solid foundation for specific detection and assessment of intervertebral disc degeneration at the molecular level and will promote development of future disc regeneration strategies.

Graphical Abstract

Keywords

intervertebral disc degeneration; collagen; molecular detection; collagen hybridizing peptide

INTRODUCTION

Over 100 billion dollars are spent every year as a consequence of low back pain, which has a lifetime prevalence of 70–85%, making it a leading cause of disability among those above the age of 45.^{1,2} Intervertebral disc (IVD) degeneration contributes predominantly to low back pain.³ Despite the many molecular and cell therapies under investigation, there is no effective therapy to treat this condition so far. IVD by nature is a “donut-shaped” connective tissue adjoining two vertebrae providing a cushion effect for different spine motions and composed of a gelatinous nucleus pulposus (NP) in the center with a lamella fibrocartilage annulus fibrosus (AF) surrounding it. Cartilaginous end plates connect the disc to spinal vertebrae.⁴ Aging and gradual wear and tear have been implicated as the most common causes of disc degeneration, although a variety of risk factors, such as inflammation, injury, and obesity, have been identified.^{5–10} Regardless of its specific etiology, disc degeneration shares a common series of morphological and biochemical changes including diminished disc height, loss of hydration in the NP, increased clefts and fissures in the AF and NP, altered phenotype of disc cells, and degradation of extracellular matrix (ECM) proteins such as collagen and proteoglycan.^{7,11}

It has been a critical and challenging task to detect and assess degradation of the ECM, a crucial component controlling the progression of tissue remodeling in many pathological conditions including IVD degeneration. The ECM in IVD is composed of many biological molecules, such as fibrillary collagens in highly organized networks that provide tensile strength and proteoglycans that promote swelling pressure.¹² AF tissue composition is largely fibrocartilaginous, with a gradual shift from abundant type I collagen in fibrous lamellae in the outer AF and to abundant type II collagen in the inner AF. Largely oriented in concentric sheets, the structural matrix components are essential for the AF to retain the structure of the NP in the center of the disc and support tensile stress.

Despite the location and subtype, collagens share a triple-helical supersecondary structure with three polypeptide chains that unfold at body temperature following initial protease cleavage.^{13–15} Conventional methods to detect collagen include histological staining and immunohistochemistry (IHC). Specifically, histological staining of collagen relies on strong binding between ionic dyes with collagen proteins, such as Picrosirius red, which stains all collagens irrespective of sub-type or structural integrity.^{16,17} IHC, however, distinguishes between specific types of collagen by recognizing an epitope on the protein but typically requires tedious hands-on operation and optimization of protocols. Earlier studies reported IHC work using in-lab-developed antibodies to detect sub-types of denatured collagen in rat articular cartilage, rat long bone, and equine articular cartilage; however, these antibodies have not been widely available.^{18–23} When lacking detailed information regarding collagen integrity at the microscopic and molecular level, it is impossible to accurately assess the functional efficacy of any newly developed reparative and regenerative strategies which seek to tackle intervertebral disc degeneration. Molecular detection of extracellular matrix components, such as collagen, within the intervertebral disc not only plays a significant role in understanding disc biology and pathology but also possesses an indispensable role as a stand-alone methodology study to meet such needs in current disc research.

To address this problem, we aim to establish a simple and quantifiable approach to assess early degenerative change in intervertebral discs with a collagen hybridizing peptide (CHP) that binds specifically to the dissociated triple helix structure in degraded collagens through a unique helix hybridization (Figure 1a). CHPs are a class of peptides with a triple-helical structure comprised of a repeated Gly-X-Y amino acid sequence. They have been shown to specifically bind to collagens degraded or destroyed by a variety of factors including enzymatic digestion, heat denaturing, and mechanical injury via triple helix hybridization.^{13–15,24,25} The CHP method, outlined in Figure 1b, provides a number of advantages over common histological methods, such as operational simplicity and protocol efficiency (Figure 1c).

Considering the needs of a globally aging population with widespread low back pain, it is imperative to understand the biology of age-associated IVD degeneration, particularly ECM remodeling, in order to develop therapeutic interventions to combat this debilitating condition.^{5,26} We first tested the feasibility of our method in detecting and characterizing collagen degeneration by analyzing mouse lumbar discs from various age groups. In brief, a facile three-step staining was conducted using CHP-FITC (F-CHP) (5 μ M) on cryostat disc tissue sections (5 μ m) from five age groups of B6 mice (both female and male): 2–3 months

($n = 21$ discs), 6–8 months ($n = 18$ discs), 10–12 months ($n = 19$ discs), 16–18 months ($n = 24$ discs), and 20–24 months ($n = 28$ discs). On average, the CHP fluorescence intensity increased progressively with increased mouse age, indicative of spontaneous disc degeneration due to aging (Figure 2a,b). Outer AF (oAF) regions exhibited much higher fluorescence than inner AF (iAF), suggesting more severe collagen breakdown in the oAF. A significant positive linear correlation was found between weighted age group (mean age of all samples in an age group) and both CHP fluorescence intensity of inner AF (iAF) ($r^2 = 0.9200$) and outer AF (oAF) ($r^2 = 0.9713$) (Figure 2c). The regression linearity of oAF fluorescence intensity was shifted upward with respect to that of iAF. Additionally, a strong positive linear correlation was found between sample age and CHP signal ($r^2 = 0.9702$) when samples were analyzed as a whole as opposed to separately by the AF region (Figure 2d). It was of note that autofluorescence signal was negligible with the chosen imaging settings (Figure S1). NP tissue was not used in this fluorescence intensity analysis due to weak signal (Figure S2).

Inflammation plays a significant role in the pathophysiological process IVD degeneration.^{9,16,27} ECM breakdown products, especially low molecular weight fragments, can induce inflammatory responses promoting macrophage mediated production of IL-1 β and tumor necrosis factor alpha (TNF- α).^{28,29} Lipopolysaccharides (LPS) are characteristic components of the cell wall of Gram negative bacteria. Both LPS and inflammatory cytokines, such as IL-1 β , can promote matrix degradation and macrophage infiltration, exacerbating degenerative conditions in discs. However, it is extremely difficult to clarify the overall role of ECM proteins within an immune setting due to their complex functions and simultaneous presence with proteinases.⁹ A lack of effective techniques by which to detect ECM degradation products alongside a limited knowledge of the pathological process of disc degeneration further exacerbated this difficulty in elucidating the role of ECM proteins. We thus explored the efficacy of our CHP method in detecting disc degeneration induced by interleukin-1 β and LPS in an *ex vivo* organ culture. Much stronger fluorescence signals were detected in LPS and IL-1 β treated discs compared to a native control (2–3 months old mouse lumbar discs) (Figure 2e) that showed weak fluorescence signal. A one-way ANOVA indicated a significant difference among means of treatment groups and controls ($***p < 0.001$) (Figure 2f). We further conducted a blocking study with excessive non-fluorescent CHP on inflammatory discs. Significantly less fluorescence ($***p < 0.001$) was observed in the blocking group compared to the non-blocked F-CHP group in both IL-1 β and LPS treated disc ($n = 5$ per group), confirming specific CHP binding to degraded collagen induced by inflammation (Figure S3). We therefore confirmed that inflammatory stimuli induced degeneration serves as a suitable *ex vivo* disc degeneration model. These results demonstrated the utility of the CHP method of molecular detection of degraded collagen as a robust means by which to assess disc degeneration incited by different factors, such as aging and inflammation.

When confirming validity of CHP method, we attempted to establish a mathematical correlation between CHP signals visualizing degraded collagen and conventional histological scoring. Disc degeneration was scored using an established method of histological analysis with alcian blue/picrosirius red staining developed by Tam et al.^{30–33} Each disc sample was scored based on four categories: NP structure, NP clefts/ fissures, AF

structure, and AF clefts/fissures. As shown in Figure 3a, with increasing age, the inner and outer AF lost structural cohesion between collagen lamellae and exhibited increased presence of clefts and fissures. NP structure appeared similar in age groups up through 10–12 months in terms of cellularity but exhibited structural deterioration such as increased severity of clefts and fissures and cell morphology change in samples older than 16 months. This age-associated disc degeneration in cryostat mouse discs was consistent with a supplemental analysis conducted in a set of paraffin-embedded mouse disc samples (Figure S4). Significant differences among means of overall histological scores were obtained among all five age groups ($**p < 0.01$, $***p < 0.001$ between two adjacent age groups) (Figure 3b). A strong positive linear correlation was found between mean histological degenerative score and CHP fluorescence intensity ($r^2 = 0.9478$) using weighted age groups (Figure 3c). Further, we asked whether the calculated linear prediction equation could effectively predict, given a sample's fluorescence value, the histological score of degenerative discs treated with inflammatory factors. IL-1 β and LPS treated discs exhibited notably decreased collagen lamella cohesion and increased presence and severity of AF clefts and fissures (Figure 3d) with this equation. As demonstrated in Figure 3e, predicted values are slightly lower than actual values, which was likely caused by two possible factors: (a) The tabulated linear correlation equation was gleaned from age-related degeneration data: $\text{Score} = 20.07 \times \text{FL} - 11.14$ (eq 1), where Score is histological score of alcian blue/picrosirius red staining and FL is mean fluorescence intensity of a particular disc tissue. (b) Disc degeneration induced by LPS and IL-1 β at one time and one dose within *ex vivo* culture microenvironment might follow a slightly varied trend mathematically from aging related degeneration. Deciphering the molecular mechanism behind age-dependent and inflammation-associated disc degeneration has emerged as a critical task in disc research.³⁴ Thus far, our approach for the first time provided a simple *in vitro* molecular detection methodology specific to degraded collagen, which would simultaneously enable visualization of ECM remodeling in a spatiotemporal manner and establish a mathematical linear correlation for quantitative assessment and prediction of disc degeneration. Since our method dramatically shortened hands-on assay time (20–30 min) and enhanced assay specificity (for degraded collagen) in disc tissue, it could ultimately accelerate design and evaluation of therapeutic and regenerative strategies *in vitro* to tackle intervertebral disc diseases.

Mice offer several advantages as a model for human disc aging and degeneration since they are geometrically and proportionally the most similar to human discs among all of the animal models tested.³⁵ However, human discs exhibit differences when compared to those of animals in various dimensions, such as anatomy and biochemical composition. To test the feasibility of our CHP method in the human disc, we processed and stained human disc samples from surgical waste with CHP as well as alcian blue/picrosirius red. AF regions were located in the human disc samples using histology. Distinct signal differences of green fluorescence intensity from F-CHP peptides were observed between normal and degenerative human AF regions in both qualitative and quantitative manners. As shown in Figure 4, healthy discs exhibited higher amounts of proteoglycans and lower amounts of collagens (nonspecific with respect to collagen type) in comparison to the degenerated human IVDs (Figure 4a,c), as expected.⁷ Correspondingly, higher fluorescence signals were

visualized in degenerative human discs (predominantly AF tissue) compared to normal human discs in all 4 areas analyzed per sample type (Figure 4b,d); *** $p < 0.001$). This result suggested our method could detect the degradation of collagen triple helix structure not only in mouse discs but also in human disc samples. This implied utility of our CHP method in assessing disc degeneration in studies using human samples as well.

In summary, we, for the first time, established a simple and efficient fluorescence imaging method to specifically detect and quantitatively assess degraded collagen at the molecular level, as a novel niche to evaluate ECM integrity and severity of degeneration in animal and human discs. This method, visualizing collagen turnover in spatially and temporally defined manners, lays a solid foundation for accelerating both basic disc biology research and tissue engineering regenerative strategies due to its potentially high specificity, sensitivity, and simplicity, compared to many existing histological methods. Due to a limited source of antibody to detect denatured collagen, we were not able to do a side-by-side comparison, but we performed immunohistochemistry with an antibody detecting native type-II collagen and discovered that CHP method was more sensitive (Figure S5). We are in the process of developing a new probe for *in vivo* molecular imaging, which would potentially empower promising opportunities for clinical diagnosis of early disc degeneration, a central challenge for clinicians making informed decision on treatment strategies.²⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors are grateful to financial support in part from National Institute of Health NIAMS Grants R01AR064792 and R21AR057512, Commonwealth Health Research Board (CHRB) Grant 207-10-18, North American Spine Society (NASS), and start-up fund from Department of Orthopedic Surgery at University of Virginia.

REFERENCES

- (1). Andersson GBJ Epidemiological features of chronic low-back pain. *Lancet* 1999, 354 (9178), 581–585. [PubMed: 10470716]
- (2). Balagué F; Mannion AF; Pellisé F; Cedraschi C Non-specific low back pain. *Lancet* 2012, 379 (9814), 482–491. [PubMed: 21982256]
- (3). An HS; Anderson PA; Haughton VM; Iatridis JC; Kang JD; Lotz JC; Natarajan RN; Oegema TR; Roughley P; Setton LA; Urban JP; Videman T; Andersson GB; Weinstein JN Introduction: disc degeneration: summary. *Spine (Philadelphia)* 2004, 29 (23), 2677–2678.
- (4). Whatley BR; Wen X Intervertebral disc (IVD): Structure, degeneration, repair and regeneration. *Mater. Sci. Eng., C* 2012, 32 (2), 61–77.
- (5). Buckwalter JA Aging and degeneration of the human intervertebral disc. *Spine (Philadelphia)* 1995, 20 (11), 1307–1314.
- (6). Ohnishi T; Sudo H; Tsujimoto T; Iwasaki N Age-related spontaneous lumbar intervertebral disc degeneration in a mouse model. *J. Orthop. Res* 2017, 36 (1), 224–232. [PubMed: 28631843]
- (7). Urban JPG; Roberts S Degeneration of the intervertebral disc. *Arthritis Res. Ther* 2003, 5 (3), 120–130. [PubMed: 12723977]

- (8). Yang W; Yu XH; Wang C; He WS; Zhang SJ; Yan YG; Zhang J; Xiang YX; Wang WJ Interleukin-1 β in intervertebral disk degeneration. *Clin. Chim. Acta* 2015, 450, 262–272. [PubMed: 26341894]
- (9). Molinos M; Almeida CR; Caldeira J; Cunha C; Gonçalves RM; Barbosa MA Inflammation in intervertebral disc degeneration and regeneration. *J. R. Soc., Interface* 2015, 12 (104), 20141191. [PubMed: 25673296]
- (10). Wuertz K; Haglund L Inflammatory Mediators in Intervertebral Disk Degeneration and Discogenic Pain. *Global Spine J* 2013, 3 (3), 175–184. [PubMed: 24436868]
- (11). Loreto C; Musumeci G; Castorina A; Loreto C; Martinez G Degenerative disc disease of herniated intervertebral discs is associated with extracellular matrix remodeling, vimentin-positive cells and cell death. *Ann. Anat* 2011, 193 (2), 156–162. [PubMed: 21330123]
- (12). Feng H; Danfelter M; Stromqvist B; Heinegard D Extracellular matrix in disc degeneration. *J. Bone Joint Surg Am* 2006, 88 (Suppl 2), 25–29.
- (13). Li Y; Yu SM Targeting and mimicking collagens via triple helical peptide assembly. *Curr. Opin. Chem. Biol* 2013, 17 (6), 968–975. [PubMed: 24210894]
- (14). Li Y; Ho D; Meng H; Chan TR; An B; Yu H; Brodsky B; Jun AS; Michael Yu S Direct Detection of Collagenous Proteins by Fluorescently Labeled Collagen Mimetic Peptides. *Bioconjugate Chem* 2013, 24 (1), 9–16.
- (15). Hwang J; Huang Y; Burwell TJ; Peterson NC; Connor J; Weiss SJ; Yu SM; Li Y In Situ Imaging of Tissue Remodeling with Collagen Hybridizing Peptides. *ACS Nano* 2017, 11 (10), 9825–9835. [PubMed: 28877431]
- (16). Xiao L; Ding M; Fernandez A; Zhao P; Jin L; Li X Curcumin alleviates lumbar radiculopathy by reducing neuro-inflammation, oxidative stress and nociceptive factors. *Eur. Cell Mater* 2017, 33, 279–293. [PubMed: 28485773]
- (17). Xiao L; Hong K; Roberson C; Ding M; Fernandez A; Shen F; Jin L; Sonkusare S; Li X Hydroxylated Fullerene: A Stellar Nanomedicine to Treat Lumbar Radiculopathy via Antagonizing TNF- α -Induced Ion Channel Activation, Calcium Signaling, and Neuropeptide Production. *ACS Biomater. Sci. Eng* 2018, 4 (1), 266–277. [PubMed: 30038959]
- (18). Billingham RC; Buxton EM; Edwards MG; McGraw MS; McIlwraith CW Use of an antineoepitope antibody for identification of type-II collagen degradation in equine articular cartilage. *Am. J. Vet. Res* 2001, 62 (7), 1031–1039. [PubMed: 11453476]
- (19). Stoop R; Buma P; van der Kraan PM; Hollander AP; Billingham RC; Meijers TH; Poole AR; van den Berg WB Type II collagen degradation in articular cartilage fibrillation after anterior cruciate ligament transection in rats. *Osteoarthritis Cartilage* 2001, 9 (4), 308–315. [PubMed: 11399094]
- (20). Chen CT; Bhargava M; Lin PM; Torzilli PA Time, stress, and location dependent chondrocyte death and collagen damage in cyclically loaded articular cartilage. *J. Orthop. Res* 2003, 21 (5), 888–898. [PubMed: 12919878]
- (21). Scheven BA; Hamilton NJ; Farquharson C; Rucklidge GJ; Robins SP Immunohistochemical localization of native and denatured collagen types I and II in fetal and adult rat long bones. *Bone* 1988, 9 (6), 407–414. [PubMed: 3248204]
- (22). Rucklidge GJ; Riddoch GI; Robins SP Immunocytochemical staining of rat tissues with antibodies to denatured type I collagen: a technique for localizing areas of collagen degradation. *Collagen Relat. Res* 1986, 6 (1), 41–49.
- (23). Otterness IG; Downs JT; Lane C; Bliven ML; Stukenbrok H; Scampoli DN; Milici AJ; Mezes PS Detection of collagenase-induced damage of collagen by 9A4, a monoclonal C-terminal neoepitope antibody. *Matrix Biol* 1999, 18 (4), 331–341. [PubMed: 10517180]
- (24). Wahyudi H; Reynolds AA; Li Y; Owen SC; Yu SM Targeting collagen for diagnostic imaging and therapeutic delivery. *J. Controlled Release* 2016, 240, 323–331.
- (25). Bennink LL; Li Y; Kim B; Shin IJ; San BH; Zangari M; Yoon D; Yu SM Visualizing collagen proteolysis by peptide hybridization: From 3D cell culture to in vivo imaging. *Biomaterials* 2018, 183, 67–76. [PubMed: 30149231]

- (26). Illien-Junger S; Grosjean F; Laudier DM; Vlassara H; Striker GE; Iatridis JC Combined anti-inflammatory and anti-AGE drug treatments have a protective effect on intervertebral discs in mice with diabetes. *PLoS One* 2013, 8 (5), No. e64302. [PubMed: 23691192]
- (27). Xiao L; Ding M; Zhang Y; Chordia M; Pan D; Shimer A; Shen F; Glover D; Jin L; Li X A Novel Modality for Functional Imaging in Acute Intervertebral Disk Herniation via Tracking Leukocyte Infiltration. *Mol. Imaging Biol* 2017, 19 (5), 703–713. [PubMed: 28050750]
- (28). Morwood SR; Nicholson LB Modulation of the immune response by extracellular matrix proteins. *Arch. Immunol. Ther. Exp* 2006, 54 (6), 367–374.
- (29). Risbud MV; Shapiro IM Role of Cytokines in Intervertebral Disc Degeneration: Pain and Disc-content. *Nat. Rev. Rheumatol* 2014, 10 (1), 44–56. [PubMed: 24166242]
- (30). Rowas SA; Haddad R; Gawri R; Al Ma'awi AA; Chalifour LE; Antoniou J; Mwale F Effect of in utero exposure to diethylstilbestrol on lumbar and femoral bone, articular cartilage, and the intervertebral disc in male and female adult mice progeny with and without swimming exercise. *Arthritis Res. Ther* 2012, 14 (1), R17. [PubMed: 22269139]
- (31). Yang F; Leung VY; Luk KD; Chan D; Cheung KM Mesenchymal stem cells arrest intervertebral disc degeneration through chondrocytic differentiation and stimulation of endogenous cells. *Mol. Ther* 2009, 17 (11), 1959–1966. [PubMed: 19584814]
- (32). Ohta R; Tanaka N; Nakanishi K; Kamei N; Nakamae T; Izumi B; Fujioka Y; Ochi M Heme oxygenase-1 modulates degeneration of the intervertebral disc after puncture in Bach 1 deficient mice. *Eur. Spine J* 2012, 21 (9), 1748–1757. [PubMed: 22832873]
- (33). Furukawa T; Ito K; Nuka S; Hashimoto J; Takei H; Takahara M; Ogino T; Young MF; Shinomura T Absence of biglycan accelerates the degenerative process in mouse intervertebral disc. *Spine (Philadelphia)* 2009, 34 (25), E911–917.
- (34). Vo N; Seo H-Y; Robinson A; Sowa G; Bentley D; Taylor L; Studer R; Usas A; Huard J; Alber S; Watkins SC; Lee J; Coehlo P; Wang D; Loppini M; Robbins PD; Niedernhofer LJ; Kang J Accelerated Aging of Intervertebral Discs in a Mouse Model of Progeria. *J. Orthop. Res* 2010, 28 (12), 1600–1607. [PubMed: 20973062]
- (35). O'Connell GD; Vresilovic EJ; Elliott DM Comparison of animals used in disc research to human lumbar disc geometry. *Spine (Philadelphia)* 2007, 32 (3), 328–333.

Figure 1.

Mechanistic illustration of a robust approach for detection and assessment of intervertebral disc degeneration using a collagen hybridizing peptide (CHP). (a) Disc degeneration, which can be induced by a range of factors (aging, inflammation, etc.), results in numerous morphological and biochemical changes. These include loss of cell and structural integrity as well as degradation of extracellular matrix proteins (e.g., collagen). Clefts become more common and severe in the AF and NP regions of the disc. During the degenerative process, the triple-helical structure of healthy collagen exhibits damage or dissociation, allowing strong and specific binding with CHP-FITC via unique helix hybridization. (b) Our method encompasses a simple three-step staining protocol (i) heat activation of CHP, (ii) probe incubation, (iii) removal of unbound probe, which permits facile detection of degenerative collagen in disc tissue samples (both human and animal). (c) This method possesses notable advantages over existing methods in terms of operational simplicity and protocol efficiency for denatured collagen staining on tissue slices.

Figure 2.

Simple and robust new method to detect early degenerative change in the mouse intervertebral disc provoked by aging and inflammation. (a) Representative images of CHP-FITC stained mouse lumbar discs shows increased fluorescence signal with aging in three of the five experimental groups in the study. (b) Quantitative analysis of fluorescence intensity in five age groups. A one-way ANOVA indicated a significant difference among fluorescence intensity means of all five age groups ($p < 0.0001$). Multiple comparison tests also indicated such distinction between two adjacent groups (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$). (c) A significant positive linear correlation was found between CHP fluorescence intensity (arbitrary units) and both inner AF fluorescence ($r^2 = 0.9200$) and outer AF fluorescence ($r^2 = 0.9713$). (d) A significant positive linear correlation was found between weighted age group (mean age of all samples in an age group) and CHP fluorescence intensity of individual disc samples ($r^2 = 0.9702$). (e) Representative images of CHP stained mouse lumbar discs of native control samples (2–3 months old) and discs subjected to IL-1 β (10 ng/mL) and LPS (100 ng/mL) treatment for 3 days. Inflammatory stimuli significantly increased collagen breakdown. (f) Quantitative analysis confirmed significant difference among means of IL-1 β and LPS treatment groups compared to control (*** $p < 0.001$). Scale bars represent 100 μm . iAF, inner annulus fibrosus; oAF, outer annulus fibrosus. All images were taken at 200 $\times$ magnification with 1 s exposure time and 3.4 $\times$ analog gain. Average fluorescence intensity values were calculated from two 100 $\times$ 100 μm^2 regions of interest for each sample. Overall, the average fluorescence intensity value was the mean of the inner and outer AF fluorescence intensities.

Figure 3.

Establishment of robust correlation between CHP-collagen method and histological scoring on severity of mouse intervertebral disc degeneration. (a) Representative images of alcian blue/picrosirius red staining of mouse lumbar disc samples including inner AF (iAF), outer AF (oAF), and NP for each age group. With increasing age, loss of structural cohesion between collagen lamellae and increased clefts and fissures were observed in inner and outer AF. NP structure was comparable in terms of cellularity for age groups up through 10–12 months and exhibited increased deterioration evidenced by increased presence and severity of NP clefts and fissures in samples 16 months and above. (b) Histological analysis using aforementioned established methods revealed a positive correlation between severity of disc degeneration and aging. A one-way ANOVA indicated a significant difference among means of histological degeneration scores in all five age groups ($p < 0.0001$), which was supported by multiple comparison tests ($***p < 0.001$; $**p < 0.01$) again between two adjacent age groups. (c) A robust positive linear correlation ($\text{Score} = 20.07 \times \text{FL} - 11.14$ eq 1) was established between mean histology score and mean CHP fluorescence intensity ($r^2 = 0.9478$), when weighted age groups (mean age of all samples in one age group) were used. (d) Representative images of untreated control disc samples and LPS and IL-1 β (3 day treatment) treated disc samples. Compared to native control (2–3 months), LPS/IL-1 β

treated samples were significantly more degenerated with notably decreased collagen lamella cohesion as well as increased presence and severity of clefts and fissures. (e) Mean degeneration scores of IL-1 β and LPS groups obtained experimentally via established histology and through theoretical prediction using the linear regression equation in Figure 3c. Scale bars = 100 μ m.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4.

Clinical manifestation of CHP method for distinct qualitative and quantitative differentiation between normal and degenerative human discs. (a) Representative images of healthy (top) and degenerated (bottom) human intervertebral disc samples stained with alcian blue and picrosirius red. Red indicates collagen, while blue indicated proteoglycan. The solid vertical column at the left side of each image was identified as end plate (EP) while the remaining tissue to the right was identified as AF. Four regions of interest were outlined (areas 1–4) for fluorescence analysis on adjacent CHP-FITC stained disc sections. Images were taken at 100× magnification. (b) Representative fluorescence image of CHP stained human disc exhibited much higher fluorescence signal visibly in a degenerative human disc tissue compared to a normal human disc (200× magnification with 1 s exposure time and 3.4× analog gain). Average fluorescence intensity was recorded for each of these regions for later analysis. (c) Representative images of the alcian blue and picrosirius red stained human disc indicated less proteoglycan (blue) and more collagen content (red) in degenerative human disc compared to healthy sample (200× magnification). (d) Quantitative assessment of CHP fluorescence signal exhibited significantly higher signal in degenerative human disc compared to that of healthy one (*** $p < 0.001$ by a Student's t -test). $n = 6$ sections per sample were analyzed.