Investigating the Chondroprotective Nature of Extracellular Matrix Protein Matrilin-3

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Thesis

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Publications

SCIENTIFIC PAPERS

Zeid, M., J.K. Yu, I. Goldowitz, M.E. Denton, D. E. Costich, **C.T. Jayasuriya**, M. Saha, R. Elshire, D. Bencher, F. Breseghello, J. Munkvold, R.K. Varshney, G. Belay, and M.E. Sorrells. 2010. Cross-amplification of EST-derived markers among 16 grass species. *Field Crops Research 118: 28–35*.

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TEXTBOOK CHAPTERS

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NATIONAL ABSTRACTS

Jayasuriya, C.T., Zhou, F., Chen, Q. 2010. ECM Protein MATN3 Shows Potential to Specifically Bind Catabolic Inflammatory Cyto/Chemokines. Poster Presentation. 56th Annual Meeting of the Orthopaedic Research Society (ORS).

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Chen, Y., Koopman, K., **Jayasuriya, C.T.,** Cossman, J., Webster, T.J., Fenniri, H., Chen, Q. 2012. Development of Novel Chondroprotective Matrilin-3 Hybrid Nanotubes that Enhance Chondrocyte Adhesion, Increase Matrix Synthesis, and Reduce Catabolic Gene Expression. 58th Annual Meeting of the Orthopaedic Research Society (ORS).

Jayasuriya, C.T., Zhengke, W., Chen, Q. 2012. Osteoarthritis associated mutation in MATN3 gene abolishes IL-1Ra dependent stimulation of chondrogenesis markers in chondroprogenitor cells. 59th Annual Meeting of the Orthopaedic Research Society (ORS).

Patents

Compositions and Methods for the Treatment of Orthopedic Disease or Injury (Filed and acquired, 2012)

Honors & Awards

Dr. Henry M. Wisniewski Award in Scientific Achievement, 2001
Presented by the Institute for Basic Research of Staten Island New York. This award is presented to 5 students who are conducting research at the Institute of Basic Research that have submitted top abstracts. Award is accompanied by a check for 500 dollars.

Certificate of Honor, 2002
Presented by New York City at the Eleventh Annual New York Science Talent Search. This award is presented to semifinalists of the annual scientific poster presentation competition.

Neuroscience Research Prize Competition Finalist, 2002
Presented by the American Academy of Neurology. Finalist status is awarded to the top 10 applicants of this annual competition.

Hughes Research Scholarship, 2005
Presented by Cornell University. This is a scholarship to cover tuition and board to participate in the prestigious Hughes Summer Research Program at Cornell University. This program's main focus is to teach its students how to articulate their research more clearly.

Pathobiology Poster Competition: 1st Place Prize, 2010
Presented by the Pathobiology PhD Program of Brown University. This award is granted to the top poster presentation at the 2010 Pathobiology Retreat. This retreat is attended by all students and faculty of the Pathobiology PhD Program. Award is accompanied by a check for 300 dollars.

NIA Grants Technical Assistance Workshop Travel Award, 2010
Presented by the National Institute of Aging (NIA) of NIH. This was a travel award given to cover costs of attending the NIA Grants Technical Assistance Workshop hosted in New Orleans, Louisiana. The annual workshop focuses on guiding grant/fellowship applicants through the selection process for NIH awards and it also helps to improve their future applications to the NIA.

Brown Pathobiology Admissions Weekend, 2011
Invited by Brown University. The Pathobiology Admissions Weekend is designated to help incoming students to have an understanding of the caliber of research conducted by Brown Pathobiology PhD students. I was one of the four students nominated by faculty to present my research at this event.

ECM in Health and Disease Symposium Travel Award, 2011
Invited by Harvard Medical School. A select number of applicants, myself included, who were scheduled to give oral presentations were awarded the travel award based on the quality of research abstracts submitted.

Nathan Shock Center Conference on Aging Travel Award, 2011
Presented by the University of Texas and NIA. This is an annual conference held in San Antonio, Texas that focuses on inflammation during aging. I presented a poster of my research.

19th Annual Hospital Research Competition: Finalist, 2011
Presented by Rhode Island Hospital. I was one of the 5 finalists selected to receive this award. The competition is open to all researchers at Rhode Island Hospital.

Webster Jee Young Investigator Award Recipient, 2012
Presented by the International Chinese Hard Tissue Society (ICHTS). This prestigious award is presented to a hand full of young investigators every year based on the merit of their scientific research on skeletal tissue.

New Investigator Recognition Award (NIRA) Recipient, 2012
Presented by the Orthopaedic Research Society (ORS). This nationally recognized award is presented to only 10 new investigators each year based on the merit of their scientific research in the field of orthopaedics.

20th Annual Hospital Research Competition: Winner, 2012
Presented by Rhode Island Hospital. I was selected as the winner of the New Investigator Award in basic science category. The competition is open to all researchers at Rhode Island Hospital.

Orthopaedic Research Society (ORS): Podium Speaker, 2013
Invited to give a podium presentation on my research at the ORS National conference. I was selected based on the merit of my 2013 abstract.

Brown Pathobiology Admissions Weekend, 2013
Invited by Brown University. The Pathobiology Admissions Weekend is designated to help incoming students to have an understanding of the caliber of research conducted by Brown Pathobiology PhD students. I was invited once more by faculty to present my graduate research at this venue.

Fellowships

Orthopaedic Foundation Pre-doctoral Fellowship, fiscal year 2010
Presented by Rhode Island Hospital. This fellowship is awarded yearly to one student conducting orthopaedics research who has excelled in academic performance and scientific achievement. The fellowship covers 1 year of graduate school tuition costs from the date that it was awarded.

Invited Talks

"**Investigating the Chondroprotective Nature of Cartilage ECM Protein, Matrilin-3,"** NIA Grants Technical Assistance Workshop 2010 in New Orleans, Invited by NIA/NIH

"Investigating the role of Matrilin-3 in cartilage homeostasis," Pathobiology Program Admissions Weekend 2011, Invited by Brown University.

"Protecting aging joint from degeneration: a novel anti-inflammation role of an ECM protein," Invited by Providence Area Aging Research Forum in 2012.

"Investigating the regulatory function of Matrilin-3 in cartilage tissue," Pathobiology Program Admissions Weekend 2013, Invited by Brown University

Teaching & Certifications

Brown University: BIOL 1540 Molecular Genetics, Teaching Assistant, Spring 2010

Teaching Certificate I recipient, Certified by Harriet W. Sheridan Center for Teaching and Learning, Spring 2012

Mentoring

Graduate mentor of **Yale University Undergraduate** Hughes Scholar, Tiffany Bell, Summer 2011

Graduate mentor of **Brown University Undergraduate** student, Kaitlyn Tracy, Spring 2012

Graduate mentor of **Brown University Undergraduate** thesis student, So Yun Hur, Fall 2012 - Present

Graduate mentor of **University of Rhode Island (URI) Undergraduate**, Nicholas Lemme, Spring 2013

Research Experience

Institute for Basic Research, Staten Island, NY (2001 – 2002)
Primary research involved analyzing the effect of retinoic acid (RA) on Herpes Simplex Virus (HSV-1) proliferation and virus induced cell death in primate kidney cells using both electron microscopy and histology. I conducted this independent research at the New York State Institute for Basic Research as a high school intern and this was my first exposure to biological research. Conclusions drawn from this study suggested that RA deterred HSV-1 viral proliferation in a dose dependent manner.

Cornell University Dept. of Plant Breeding and Genetics (2003 – 2006)
Primary research involved identifying linkage groups in grass species using comparative genomics and polymorphic markers. I became proficient in Real-time PCR, polyacrylamide gel electrophoresis, gel staining/photography, western blot, and DNA extraction from plant matter. Conclusions drawn from this study allowed for the identification of conserved linkage groups that exist between grass species that currently have very few available molecular markers.

Weill Cornell Medical School (2006 – 2008)
During my time working as a GMP technician in the Gene Therapy Core Facility at Weill Cornell Medical School in New York City, I was assigned to optimize laboratory protocols to enhance AAV viral output from transfected HEK 293 cells for the purposes of gene therapy. Conclusions drawn from this study allowed for the

optimization of both transfection efficiency and resulting viral load by more than 40% in comparison to pre-existing laboratory protocols.

Brown University Dept. of Orthopaedics Research (2009 – Current)
Currently I am earning my Ph.D. in the Orthopaedics Department at Brown University under my advisor and mentor, Qian Chen. My ongoing thesis work involves identifying the biological mechanism underlying matrilin-3's potential to regulate cartilage homeostatic processes, which we have found to contribute to its overall ability to act as a chondroprotective ECM protein which is necessary for maintaining cartilage health during aging. This work is a major component of my thesis project. I am also a collaborator on a secondary project focused on identifying the significance of matrilin-3 in growth plate chondrocytes during chondrogenic cell development. This work is all focused on the use of matrilin-3 in combination with biomaterials to enhance cartilage tissue engineering for the purpose of repairing joint injury and/or treating joint disease.

Organizational Memberships

- Orthopaedic Research Society (ORS)
- American Association of Anatomists
- ACACIA Fraternity (Cornell Chapter)

Preface

I, C. T. Jayasuriya, have designed the experiments included in this dissertation with the guidance of my mentor Q. Chen (PhD). I have executed all experiments included in this dissertation with the following exceptions:

Primary human cartilage tissue was isolated surgically from patients of Rhode Island Hospital by Richard Terek (MD) and Richard Limbird (MD).

In Chapter 3, F. Zhou (PhD) of the Chen Laboratory assisted in the acquisition of data by performing confocal microscopy of stably transfected ATDC5 cell line phenotypes.

In Chapter 3, M. Pei (PhD) of West Virginia University, Morgantown, WV contributed to acquisition of data by performing safranin-O staining and dimethyl-methylene blue staining of primary porcine synovial fibroblasts (PSFBs).

In Chapter 3, P. Haines (MS) of the Chen Laboratory contributed to the acquisition of Western blot and immunohistochemistry staining used to characterize the stably transfected ATDC5 cell lines.

In Chapter 3, Z. Wang (PhD) of the Chen Laboratory generated the four stably transfected ATDC5 cell lines used in this study.

In Chapter 3, J. Luo (PhD) of the Chen Laboratory generated the wild-type and mutant matrilin-3 gene constructs that were used for transfecting cells in this study.

Dedication

This thesis is dedicated to my Mother-in-law, Dileeni Priyanjalie Bowatte, who passed away on 1st August 2012 of colorectal cancer. You were the strongest person we knew and you will be loved and missed always.

Acknowledgements

To my PhD thesis advisor, mentor and friend Dr. Qian Chen: Thank you for always being in my corner and believing in me when no one else would. I am forever grateful for the confidence that you have given me and the strong work ethic and enthusiasm for science that you have helped me to develop throughout the years. I will always look up to you as a shining example of the kind of scientist I aspire to be.

To the past and present members of the Chen laboratory: Thank you for being my second family. None of this would have been possible without the lessons that I have learned from you.

To my PhD Thesis Committee (Drs. Alfred Ayala, Laurent Brossay, Chanika Phornphutkul and Mary Goldring): Thank you for guiding me with your wisdom and experience. I owe my development as a scientist to you all.

To senior students (now Alumni) of the Brown Pathobiology and MCB PhD programs that have advised me throughout the years, especially Drs. Leon Toussaint, Kevin Alby, Pamela Gaddi, Meredith Crane and Racquel Sherwood: I cannot find the words to express my gratitude for the guidance that you have given me. Even while having no obligations whatsoever to listen to my problems and concerns you have made time to do so and for that I am eternally grateful. On this note, Leon only you and I will know what this means but McGlider tells me that you have made his book…. Now go celebrate.

To the Brown Pathobiology PhD program including its administrators, staff and faculty: Thank you for giving me the chance and academic structure to rediscover why I love science.

To my loving wife Sonalya, who has been my anchor for the past 5 years: Your love and support has helped me become a much better man today than I was before I met you. For this, I owe much of my success in everything to you.

To my Mom and Dad: Thank you for sacrificing so much for the sake of my education and for always being there for me when I needed you.

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ABSTRACT

Matrilin-3 (MATN3) is a member of the matrilin family of extracellular matrix (ECM) proteins. It is specifically found in cartilage where it is classically believed to function as a structural protein that physically stabilizes tissue integrity. Mutations in the MATN3 gene are associated with a variety of skeletal diseases with varying severity including chondrodysplasia and hand osteoarthritis (HOA). Furthermore, a recent study has shown that mice deficient in the functional MATN3 gene appear to undergo relatively normal skeletal development, yet they exhibit higher and earlier incidences of osteoarthritis (OA). Therefore, normal MATN3 prevents OA pathogenesis *in vivo*. Currently it is not known how MATN3 protects cartilage tissue from OA; however, the present study shows for the first time that MATN3 can regulate certain cartilage homeostasis markers. This study utilizes gene expression and protein analysis to investigate the underlying biology that confers chondroprotectivity of MATN3 by investigating its effects on both mature chondrocytes and mesenchymal stem cells (MSCs) of the chondrogenic lineage. It also employs PI3K and EGFR inhibitor studies to sheds light on key cell signaling pathways that are vital for the protective role(s) of MATN3 in chondrocytes.

CHAPTER 1

BACKGROUND AND SPECIFIC AIMS

This chapter includes sections taken directly from:

Cartilage Extracellular Matrix Integrity & OA

C.T. Jayasuriya and Q. Chen

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Osteoarthritis – An ever prevalent chronic joint disease

Today people live longer due to advances in medicine. Along with this blessing comes the ever-increasing prevalence of age-associated disease. In the United States alone, more than 33% of the population over the age of 65 suffers from Osteoarthritis (OA) [1]. In 1992, it was estimated that OA cost our country over 65 billion dollars per year. As of today, this number has more than doubled making OA currently the most common disability-causing arthropathy worldwide among middle aged to elderly populations. With the exception of surgical intervention, which can often be drastic (i.e., complete joint replacement), there is no effective way to permanently alleviate OA-induced pain. Trying to manage with this disease can also be extremely difficult as the effects of commonly used non-steroidal anti-inflammatory drugs and corticosteroids are typically short-lived, providing limited efficacy.

OA is a chronic joint disease and takes time to develop. Chronic injury and extensive mechanical loading of diarthrodial joints due to extreme physical activity and/or obesity are leading factors that result in sustained damage to articular cartilage which can lead to OA development. The pathophysiology of OA has been studied extensively; however, the exact cause of this disease remains to be elucidated and consequently there is no FDA approved drug specifically designated for its treatment. Although not considered a classical inflammatory arthropathy, OA is frequently associated with signs and symptoms of inflammation, including joint pain, swelling/stiffness which eventually leads to functional impairment and, in extreme cases, disability [2]. It is believed that

dysregulation of chondrocyte function due to physical stress/injury induced inflammation causes an imbalance between catabolic and anabolic activities involved in normal turnover of the cartilage extracellular matrix (ECM) leading to increased cartilage degradation [3].

Understanding the tissue that keeps us moving smoothly

Articular cartilage is an avascular aneural connective tissue composed of a sparse population of cells called chondrocytes that secrete large quantities of ECM proteins, which are the major components of cartilage [3]. During early bone development, MSCs in a chondroprogenic environment condense and undergo differentiation into chondrocytes, which proliferate and mature. Some of these cells eventually undergo terminal differentiation, where the matrix remodels and calcifies, and undergo cell death as the tissue is replaced by bone. This process leaves behind a layer of articular cartilage on the epiphyseal surfaces of bones providing a low friction surface that can act as a weight/shear stress-bearing coat allowing for smooth joint transition during movement.

Although articular cartilage is anatomically classified as a single tissue type, it is divided into four zones defined by their positions relative to the joint surface. Likewise, the populations of chondrocytes housed within these zones and their respective ECMs often differ from one another in both appearance and organization (Figure 1). The calcified zone lies directly on top of the subchondral bone, which the cartilage tissue shields from physical forces. This zone contains a very small population of chondrocytes that are slowly being replaced by bone forming cells (osteoblasts) continuously throughout life. When compared to other

cartilage zones, the calcified zone ECM is highly mineralized and contains the sparsest chondrocyte population. Osteoblasts from the neighboring subchondral bone secrete bone morphogenic factor (BMPs), and other factors such as stromal cell derived factor 1 (SDF-1) which promote chondrocyte hypertrophy and mineralization [4, 5]. The deep zone cartilage layer lies directly above the calcified zone and contains small vertical aggregates of chondrocytes embedded within a uniquely organized ECM which histologically resemble columnar structures. The middle zone is by far the largest layer containing rounded chondrocytes and a well-hydrated and robust collagen ECM network. Chondrocyte content increases gradually from the subchondral bone towards the articular surface that is in direct contact with the joint synovial fluid. The superficial zone (A.K.A. tangential zone) makes up the articular surface and therefore contains the largest number of chondrocytes of all four zones.

Since articular cartilage consists largely of water and ECM, which minimizes surface friction and supports suppressive forces during joint loading, the three main individual constituents of this ECM network are collagens, proteoglycans, and non-collagenous matrix proteins [3]. Several collagens are cartilage specific including type II, VI, IX, X, and XI [3, 6]. However, type II is the major collagen component and makes up 90 - 98% of total tissue collagen in the articular cartilage of humans. Type II collagen and other collagens form aldimine (carbon nitrogen double-bonded) crosslinks that are condensed into fibrils which provide the tensile strength necessary for articular cartilage to perform its load-bearing function [7]. This collagen network is resistant to both mechanical and

thermal dissociation and therefore allows cartilage to withstand load and shearing forces that commonly arise from movement of joints. Proteoglycans, specifically aggrecan, are the second major component of articular cartilage [8]. These heavily glycosylated proteins have multiple glycosaminoglycan (GAG) monomers bonded to a core protein chain which spans the ECM [9]. Aggrecan is composed of GAG monomers bound to a single hyaluronan protein chain, which intertwines with the collagen fibril network to form a tightly woven protein network that can withstand shear forces [10]. Various non-collagenous proteins such as matrilins and the cartilage oligomeric matrix protein (COMP) make up the third and smallest component of the articular cartilage ECM. Mutations in these proteins have been found to correlate with developmental abnormalities such as skeletal dysplasias and chondrodysplasias which can lead to OA development [11-15].

OA pathophysiology in cartilage – A breach in the balance

OA can affect just one or all four cartilage zones depending on the severity and pathological stage of the disease. Given its anatomical position, the superficial zone is often the first cartilage tissue zone to be exposed to injury or wear-and–tear due to excessive joint loading. Therefore, this zone often appears to be the initial point of OA pathogenesis. During early stage OA, a sustained injury to the articular surface initially induces a mild but chronic inflammatory response that slowly manifests into the disruption of cartilage homeostasis due to disredulation of chondrocyte function [16]. OA is clinically characterized by its degenerative effect on major articular cartilage ECM components [6]. In normal

healthy cartilage, ECM molecules are slowly broken down via proteolysis and replaced by newly synthesized ECM proteins secreted from nearby chondrocytes. The catabolic and anabolic processes of this turnover are balanced in normal cartilage so that the rate of proteolysis and ECM loss matches the rate of ECM synthesis [17]. However, in OA cartilage, this balance is often observed to be shifted towards catabolism. Proteases act to degrade the ECM network by cleaving excessive amounts collagen and proteoglycans [3]. These cleaved fragments are released into the cartilage matrix and some can even trigger further tissue catabolism (Figure 2) by both known and unknown biological mechanisms. The degeneration of the joint cartilage is further enhanced by disregulation of tissue repair due to disregulated anabolism. During OA, the disregulation of common anabolic growth factors native to the articular cartilage (i.e. TGF-β, FGF and IGF) prevents adequate protection against the catabolic effects induced by proteases ultimately leading to an imbalanced cartilage turnover process that favors degradation [18, 19].

Proteolytic enzymes that facilitate ECM breakdown

The enhancement of articular cartilage ECM catabolism during OA is mediated mostly by the matrix metalloproteinase (MMP) family of collagenases and the ADAMTS family of aggrecanases which are often expressed by chondrocytes in response to elevated levels of inflammatory cytokines such as IL-1β [3, 20, 21]. MMPs are neutral zinc-dependent endoproteinases that, when activated, cleave and degrade ECM components during normal tissue turnover.

The MMP family is divided into several categories based on their enzymatic activity: collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs). MMPs commonly involved in cartilage homeostasis are collagenases and gelatinases. Most MMPs are initially secreted as inactive pro-MMP proteins (zymogens) which are then activated by proteolytic cleavage themselves. Because of their catabolic activity, this family of proteases has received considerable attention in arthritis research. Both mRNA expression and enzymatic activity of certain metalloproteinase are increased in cartilage tissue during OA pathogenesis including: MMP-1 [22], MMP-2 [23, 24], MMP-3 [25], MMP-7 [26], MMP-8 [22], MMP-9 [24], MMP-10 and MMP-13 [27]. Table 1 lists OA associated catabolic proteases and the matrix protein targets that they cleave.

MMP-1, -3, -9 and -13 are of particular significance because these four proteases are the most damaging to cartilage tissue in OA. MMP-1 is classified as a collagenase that shows preference for cleaving type III and type X collagens [3, 28]; the latter is not a major component of ECM, but it is still present in articular cartilage tissue. MMP-1 is stoichiometrically inhibited by tissue inhibitor of metalloproteinase (TIMP) 1 and 2. MMP-3 is upregulated in early OA. Immunohistochemical studies have previously demonstrated that MMP-3 is expressed primarily in the superficial and transition zone in early stage OA cartilage and MMP-3 staining positively correlates with grade of OA according to Mankin scores. In addition to degrading type IX collagen and certain proteoglycans [3, 29], MMP-3 initiates a cascade that ultimately cleaves and

activates pro-MMP-1, which is a precursor to MMP-1. MMP-9 is different from the MMP-1 and MMP-3 because it is classified as a gelatinase which prefers denatured collagen, mostly type IV and V, as a substrate for its catabolic activity [25]. Its mRNA expression is minimal in normal articular cartilage, but it is greatly elevated in fibrillated areas of OA cartilage. Although many members of the MMP family are involved in cartilage ECM catabolism, no other MMP is more damaging to cartilage tissue during OA than the collagenase MMP-13 [27, 30]. Type II collagen is the primary structural component of the articular cartilage ECM for which MMP-13 shows substrate preference over any other collagen type [25, 26]. For this reason, it is the collagenase that causes the most cartilage ECM destruction during OA. In addition to type II collagen, it also cleaves type III, IV, IX and X collagen species endogenous to cartilage tissue. MMP-13 is normally synthesized in many different tissues including skin, bone, muscle, and cartilage. Its gene expression coincides with type X collagen gene expression in cartilage undergoing hypertrophic differentiation [31]. In normal healthy cartilage, the primary role of MMP-13 is to enable hypertrophic zone expansion as it denatures pre-existing type II collagen fibrils of the ECM [6, 32]. However, it has been shown that transgenic overexpression of constitutively active MMP-13 in articular chondrocytes also induces OA phenotypic changes [27]. Previous studies have attempted to use MMP-13 inhibitors such as pyrimidinetrione analogs [22] and benzofuran [33] to remedy OA induced cartilage damage. However, their responsiveness was found to be dose-dependant and often caused unwanted musculoskeletal side effects [34].

IL-1 is a key mediator of cartilage destruction in OA

Unlike RA, OA is not traditionally classified an inflammatory arthropathy; however, it is characterized by mild yet chronic inflammation that indirectly plays a significant role in disease progression and tissue destruction. Pro-inflammatory cytokine and chemokine production by mononuclear cells, cells of the synovial membrane, and articular chondrocytes can disrupt normal cartilage homeostasis favoring proteoglycan depletion and tissue destruction. Two major pro-inflammatory cytokines noted for their particularly destructive effects on cartilage destruction are IL-1β and TNF-α [35].

IL-1β is expressed and released mainly by synoviocytes and mononuclear cells during joint inflammation (Figure 2), but studies have shown that articular chondrocytes of OA cartilage also upregulate its expression and synthesis [36, 37]. IL-1β exerts several significant catabolic and anti-anabolic effects that make it the most disease causative cytokine in OA. It induces expression and release of collagenases by articular chondrocytes, especially MMP-1, MMP-3, MMP-9 and MMP-13, which are believed to contribute significantly to the enhancement of articular cartilage catabolism that occurs during OA [3]. The IL-1β pathway ultimately activates nuclear factor-κB (NFκB), which is necessary for the transcription of many genes relevant to OA and joint inflammation including MMPs [38]. It has been shown in murine articular cartilage explants that suppressing MMP production via IκB kinase inhibitiors is sufficient to reduce the degredation of both type II collagen and aggrecan [39].

The ability of IL-1β to downregulate the expression of type II collagen and aggrecan, the two main structural components of the articular cartilage ECM, further illustrates how this pathway can potentially hinder ECM repair in OA pathogenesis. It has been previously demonstrated that IL-1β induces a greater than twofold downregulation of both type II collagen and aggrecan expression in human chondrocytes [40, 41]. The production of type II collagen and aggrecan is important in chondrogenesis during which secretion of the correct ECM protein components is necessary for proper cartilage development. Even though chondrogenesis and chondrocyte terminal differentiation occur primarily during development in humans, these processes can be reactivated as a result of damage sustained to articular cartilage (as in the case of OA) [42]. IL-1β can inhibit chondrogenesis [43] by downregulating the transcription factor SOX9 [44], which is a master regulator of the chondrogenesis pathway. Similarly, IL-1β downregulates the expression of certain TIMPs that normally bind and inhibit active MMPs [3, 45, 46]. It is also known that the IL-1 receptor (IL-1RI) expression is higher in OA cartilage than in normal cartilage [47] indicating the possibility that the IL-1β pathway is more active in OA chondrocytes. IL-1RI KO mice are resistant to the early development of OA [47]. All evidence points to IL-1β stimulation as a potential cause of articular cartilage ECM breakdown during OA. This is why it may be possible to regulate IL-1β activity, perhaps through endogenous pathway inhibition, to slow down OA development/progression.

Blocking the IL-1 pathway can prevent OA damage

The IL-1β pathway has several endogenous inhibitors [3, 48]. Normal signal transduction of this pathway is initiated upon ligand binding to the IL-1 receptor. The ligand binding event enables IL-1RI to associate with another cell membrane bound protein known as the interleukin-1 receptor accessory protein (IL-1RAcP), which is necessary for pathway activation [49, 50]. The association of these two membrane-bound proteins allows for cross phosphorylation to occur in their transmembrane signaling domains initiating the signaling cascade which eventually leads to transcription of the proteases and cytokines described previously. Interleukin-1 receptor II (IL-1RII) is a cell membrane-bound protein which competes with IL-1RI for IL-1 ligand binding [51]. IL-1RII is an IL-1RI protein mimic which does not contain a transmembrane signaling domain therefore it will not initiate signal transduction of the pathway and is thus classified as an IL-1β pathway inhibitor. Two other endogenous inhibitors of this pathway are known as soluble interleukin-1 receptor II (sIL-1RII) and soluble interleukin-1 receptor accessory protein (sIL-1RAcP) [51]. These proteins mimic IL-1RI and IL-1RAcP respectively. sIL-1RII competes with IL-1RI to bind IL-1β, similarly sIL-1RAcP competes with IL-1RAcP to bind the IL-1RI.

The fifth, and arguably the most effective, inhibitor of this pathway is the IL-1RA [52]. This protein is an IL-1α/β protein mimic and binds IL-1RI with a much higher affinity than does either IL-1α or IL-1β [53]. IL-1RA-bound IL-1RI cannot associate with IL-1RAcP and therefore is unable to initiate signal transduction of the IL-1β pathway (Figure 3). The IL-1RA gene can be

alternatively spliced to form different isoforms. Currently four isoforms are known to exist in humans and two in mice [54, 55]. In humans, there are three intracellular isoforms of IL-RA (icIL-RA1, icIL-RA2, icIL-RA3) and one cell secreted isoform (sIL-1RA). The intracellular isoforms tend to be cell associated, since they stay in contact with the cell membrane of the cell that produces them. The secreted form of IL-1RA, however, can move into the extracellular space and proceed to inhibit the IL-1β signal transduction of cells that are further away. Several of these isoforms can be easily distinguished form one another due to their varying size: icIL-RA1/ icIL-RA2 (18-kDa), icIL-RA3 (16-kDa), and sIL-1RA (17-kDa) [51].

IL-1RA is produced by many cell types including articular chondrocytes [56]. It has been established that chondrocyte-derived IL-1RA protein helps sustain articular cartilage integrity during both RA- and OA-induced inflammation. The latter was demonstrated that, when chondrocytes taken from OA cartilage transduced with IL-1RA, there was protection against IL-1-induced cartilage degradation in organ culture experiments [57]. Further support for the idea that IL-1RA is chondroprotective comes from IL-1RA knockout mice of multiple genetic backgrounds, which develop early arthritis compared to wild-type mice of the same background [48]. IL-RA knockout mice bred in both BALB/cA and MFIx129 backgrounds developed severe inflammatory arthritis. Additionally, IL-1β protein levels were elevated as high as three-fold in the IL-1RA knock-out mice on both backgrounds, while detectable levels of B-cells and T-cells remained constant between IL-1RA knock-out and wild-type mice [58].

In 1999, *in vivo* IL-1RA gene transfer experiments done in rabbits also demonstrated its potential to reduce OA severity. In these experiments, OA was artificially induced in the animals via meniscectomy after which local IL-1RA gene therapy by intra-articular plasmid injection was performed at 24 hour intervals at 4 weeks post-surgery. The animals were sacrificed exactly 4 weeks after the first injection and the joint synovial tissues were dissected and stained for IL-1RA. The levels of IL-1RA present in the synovia of these rabbits positively correlated with a reduction in articular cartilage lesions that resulted from OA indicating that IL-1RA was chondroprotective [59]. A more recent study in 2005 looked at the levels of several potential chondrodestructive (IL-1α, IL-1β, TNF-α, etc.) as well as chondroprotective cytokines, one of which was sIL-1RA, in 31 patients at a high risk of developing OA in one knee due to chronic anterior cruciate ligament (ACL) deficiency. This study found that concentrations of IL-1β and TNF-α were significantly higher in the ACL deficient vs. normal knees while the concentration of sIL-1RA decreased with increasing grades of articular chondral damage [60].

It is important to note that the chondroprotective effects of IL-1RA during OA are only observable when the protein is consistently present in the synovium of the arthritic joint. This explains why short-lived drugs such as AnikinRA [61], which only lasts 4 hours after intraarticular injection into human patients (as determined by serum analysis) have limited efficacy in treating OA progression [62]. This is also most likely the underlying reason behind the success of longer lasting treatment options such as gene therapy and other methods aimed at

increasing autologous IL-1RA production within the synovium of the individual suffering from OA.

The matrilin family

The matrilins are a family of noncollagenous oligomeric ECM proteins that are found in a broad range of tissues including articular cartilage and bone [63-65]. There are currently four known members within the matrilin (MATN) family (Figure 4). MATN1 and MATN3 are cartilage specific while MATN2 and MATN4 are found in many connective tissue types [66-68]. It has been demonstrated that matrilins form a filamentous network pericellularly in the cartilage ECM [69].

MATN3 is the smallest and most recently discovered member of the matrilin family of ECM proteins. MATN3 contains a single vWFA domain, four EGF-like domains, and one alpha-helical oligomerization domain, which allows it to form homo-oligomers with other MATN3 peptides and hetero-oligomers with MATN1 [69]. MATN3 is naturally found in the articular cartilage in its tetrameric form composed of four single oligomers covalently bound together by their alpha-helical oligomerization domains.

What's so special about matrilin-3?

The functional significance of MATN3 has yet to be thoroughly investigated. However, several mutations in the MATN3 gene are closely linked to arthropathy, indicating that wild-type MATN3 has an important role in cartilage (Figure 5). These mutations can lead eventually either to OA directly, in the case of hand OA [70], or indirectly, in the case of multiple epiphyseal dysplasia (MED),

which manifests with joint pain and early onset OA [71, 72]. A threonine to methionine missense mutation (T298M) in the first EGF-like domain of MATN3 correlates with the development of hand OA [73] while a cystine to serine (C299S) missense mutation in this same region is common to many patients suffering from spondylo-epi-metaphyseal dysplasia (SEMD), which is a condition often leading to vertebral, epiphyseal/metaphyseal anomalies during development [74]. Likewise, an arginine to tryptophan missense mutation (R116W) in the vWFA domain has been associated with MED. It was discovered that this particular mutation prevents normal secretion of MATN3 from chondrocytes due to a dominant-negative interaction between mutant and normal MATN3 quickly leading to increased MATN3 retention within the endoplasmic reticulum of these cells [75]. Consequently, the reduction in the secretion of functional MATN3 is believed to contribute to MED.

Interestingly, during advanced stages of OA, the joint synovial fluid contains higher levels of cleaved ECM proteins including MATN3 oligomers due to the proteolysis of articular cartilage. One study has even shown that MATN3 mRNA is upregulated in some OA patients suggesting that the body may produce an excess of the protein [76]. Matrilin proteins are relatively well conserved between mice and humans making them ideal proteins to investigate in the mouse model. Functional deletion of the MATN3 gene in mice surprisingly results in no gross skeletal deformities at birth, but does however result in the development of OA much earlier in life [66]. MATN3 knockout mice were maintained on a C57BL/6J background and developed several signs of enhanced

OA including osteophyte formation and the spread of large lesions in the superficial zone of the articular cartilage, which is the layer that is in direct contact with the knee joint synovium. Additionally, these knockout mice appear to have higher bone mineral density (BMD) and lower overall cartilage proteoglycan content when compared to wild-type mice of the same genetic background. Perhaps the increase in BMD leads to over-loading of diarthroidial joints which eventually manifests in the form of enhanced cartilage damage. Tentatively, the ability of MATN to prevent OA-like lesion formation in articular cartilage may also be related to regulatory functions that go beyond its classically hypothesized role as a purely structural molecule. The complete biological mechanism by which this ECM protein acts to protect cartilage remains to be elucidated. Understanding its mechanism of protection may prove useful for tissue regenerative medicine and OA therapy.

Conclusion

Currently, there is no cure for OA because it is a complex joint disease that involves the disregulation of many homeostatic processes and ultimately favors cartilage tissue degradation and chondrocyte hypertrophy. Identifying the source of this disregulation is difficult because chronic injury, wear and tear due to overuse, and individual genetics all contribute, alone or in combination, to the development and progression of this disease. However, in every situation OA is harbingered by high levels of matrix-degrading proteases and the inflammatory cytokine IL-1, which effectively suppresses anabolic molecules such as type II

collagen and aggrecan which are essential for preserving ECM integrity and load bearing function of articular cartilage tissue. Recently, we have discovered that mice functionally deficient in the cartilage-specific MATN3 exhibit significantly lower expression of type II collagen and aggrecan in the articular cartilage relative to their wild-type littermates. This new finding coupled with the previous report that MATN3 -eficient animals develop early OA raises the question whether MATN3 is capable of regulating cartilage homeostasis processes that can potentially protect against the onset and progression of this prevalent joint disease. This thesis includes several studies that look closely at the potential regulatory role of MATN3 on cells of the articular cartilage tissue and elucidates the underlying biology behind its effects. Here we show new evidence suggesting that MATN3 has both anabolic and anti-catabolic properties that may be utilized to dampen OA-induced cartilage destruction. Additionally, we show that MATN3 mediates proper cartilage development by promoting chondrogenesis of progenitor cells into mature articular chondrocytes. Overall, our findings bolster our central hypothesis which states that in addition to its structural role, MATN3 also has several important regulatory roles in cartilage tissue that can explain its chondroprotective characteristics.

Specific Aims

I. Determine whether the regulation of cartilage homeostasis markers by MATN3 in articular chondrocytes is dependent on its stimulation of the anti-inflammatory cytokine: interleukin-1 receptor antagonist (IL-1Ra).

In a previous study our laboratory has shown that animals functionally deficient in the MATN3 gene develop OA lesions in their joint cartilage, suggesting that the presence of MATN3 is chondroprotective; however the molecular mechanism behind this protective effect is not fully understood. Preliminary experiments using human chondrocytes have shown us that this chondroprotective effect may, at least in part, be due to the ability of MATN3 to inhibit the expression of highly active pro-OA proteases (MMP-13, ADAMTS-4, ADAMTS-5) and the enhancement of type II collagen and aggrecan. Consistent with this finding is our discovery that mice deficient in functional MATN3 exhibit lower basal expression of type II collagen and aggrecan in their articular cartilage. Furthermore, our preliminary studies show that MATN3 can also upregulate the expression of IL-1Ra, which is an anti-inflammatory cytokine that is a natural inhibitor of the IL-1 pathway and its downstream effects that are well known to mediate OA progression. The purpose of this aim is to determine whether MATN3 is mediating its chondroprotective effects in cartilage through upregulation of IL-1RA.

In addressing this aim, we show that stimulation of IL-1Ra by MATN3 is required to maintain expression of the anabolic markers, type II collagen and aggrecan, in human articular chondrocytes. We also show that inhibition of

ADAMTS-5 expression (but not ADAMTS-4 and MMP-13 expression) by MATN3 in human articular chondrocytes is also dependent on its induction of IL-1Ra. Overall, this study shows that IL-1Ra is a key mediator of MATN3-induced effects that can protect against OA.

II. Investigate the function(s) of wild-type and mutant MATN3 during chondroprogenitor cell differentiation and determine whether such functions depend on IL-1Ra.

Recent studies show that there is a population of mesenchymal stem cells (MSCs) of the chondrogenic lineage called chondroprogenitors that exist in articular cartilage tissue alongside chondrocytes. These cells have the capacity to undergo chondrogenesis and differentiate into mature articular chondrocytes that can replenish damaged cartilage tissue and therefore are of great utility to regenerative medicine. A previous study has shown that MATN3 in conjunction with the growth factor TGF-β can induce chondrogenic differentiation of porcine synovial fibroblasts (PSFBs) *in vitro.* The study concluded suggesting that MATN3 may have a synergistic effect on TGF-β induced chondrogenesis of PSFBs by stabilizing cartilage-specific matrix structures in a way that promotes cell adhesion and aggregation, which can enhance the efficiency of cell differentiation. In this aim we wanted to investigate whether MATN3 can also act chondroprotectively by promoting chondrogenesis of chondroprogenitor cells. Furthermore, we want to elucidate the effects of known MATN3 mutations that are associated with multiple epiphyseal dysplasia (MED), spondylo epi metaphyseal dysplasia (SEMD) and hand osteoarthritis (HOA) on chondroprogenitors. Finally, we also sought to determine whether any of the functions of MATN3 were dependent on IL-1Ra, since our earlier findings showed that this anti-inflammatory cytokine is intrinsically involved with the protective effects of MATN3 on articular chondrocytes.

In addressing this aim, we show that MATN3 is capable of stimulating spontaneous chondrogenesis of chondroprogenitor cells without the help of other differentiating agents, such as TGF-β. We show that MATN3 mutations associated with MED, SEMD and HOA can, to varying degrees, hinder ability of MATN3 to maintain the chondrogenic phenotype of chondroprogenitors. And finally, we demonstrate that wild-type MATN3, but not the arthritis-associated mutant MATN3 gene, stimulates IL-1Ra expression in chondroprogenitors and that this phenomenon is necessary for maintaining important chondrogenesis markers such as type II collagen, aggrecan, and the expression of the transcription factor Sox-9.

III. Elucidate specific cell signaling events required for the regulation of cartilage homeostasis markers by MATN3**.**

Previous studies have hypothesized that MATN3 acts to stabilize the cartilage tissue by bridging the gap between small proteoglycans and larger collagen networks, and therefore has primarily a structural function. In completing the previous aims, we have discovered that MATN3 also has several regulatory properties that affect cells of the articular cartilage and that its utility goes beyond that of a purely structural protein. In this third and final aim we sought to identify cell signaling molecules that mediate the process of signal transduction that allows MATN3 to regulate cartilage homeostasis markers in chondrocytes.

In addressing this final aim, we have discovered that epidermal growth factor receptor 1 (EGFR1) activity and PI3 kinase activity are both necessary for MATN3 to regulate key chondrogenesis markers in chondrocytes. We show that MATN3 is able to signal through the PI3 kinase pathway eventually leading to the phosphorylation and activation of the MAP kinase ERK, which was previously reported to be necessary for IL-1Ra induction *in vitro*. Furthermore, we have found that MATN3 can inhibit the phosphorylation of the MAP kinase P38, which is upstream of many catabolic proteases including MMP-13, as shown in previous studies.

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TABLE 1

OA associated proteinase	Matrix Substrate
MMP-1	Types I, II, III, VII, VIII, X collagen, aggrecan
MMP-2	Types IV, V, VII, X collagen, aggrecan, decorin
MMP-3	Types II, III, IV, V, IX, X collagen, aggrecan, decorin
MMP-7	Types IV, X collagen, aggrecan, versican
MMP-8	Types I, II, III collagen, Aggrecan
MMP-9	Types IV, V collagen, Decorin
MMP-10	Types III, IV, V collagen, Aggrecan
MMP-13	Types II, III, IV, IX, X collagen, aggrecan
ADAMTS-4	Aggrecan, matrilin-3
ADAMTS-5	Aggrecan, brevican, matrilin-3

Table 1. Osteoarthritis (OA) associated MMPs and their cartilage extracellular matrix substrates.

FIGURE 1

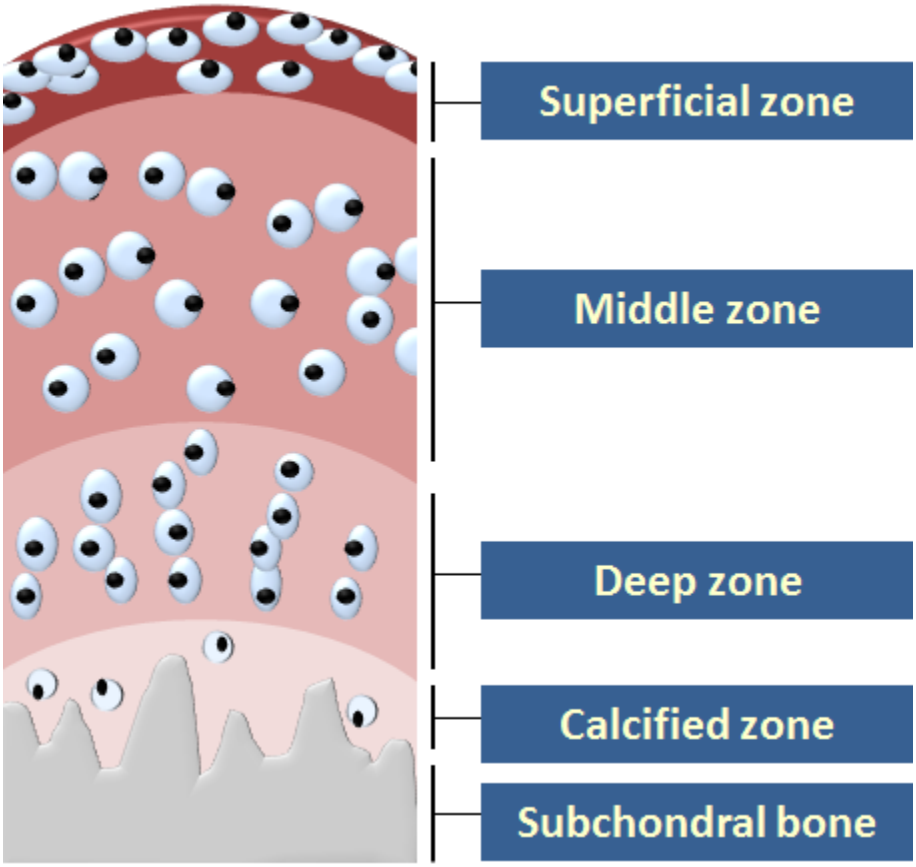

Figure 1. Articular cartilage zones. Articular cartilage consists of four layers distinguished by their position and chondrocyte subpopulations. The surface of the superficial zone is in direct contact with joint synovial fluid. This zone contains the largest number of chondrocytes per unit area. Directly underneath is the middle zone, which is the largest zone. The deep zone beneath contains chondrocytes arranged in a columnar manner. The calcified zone is the deepest layer and it contains the fewest chondrocytes.

FIGURE 2

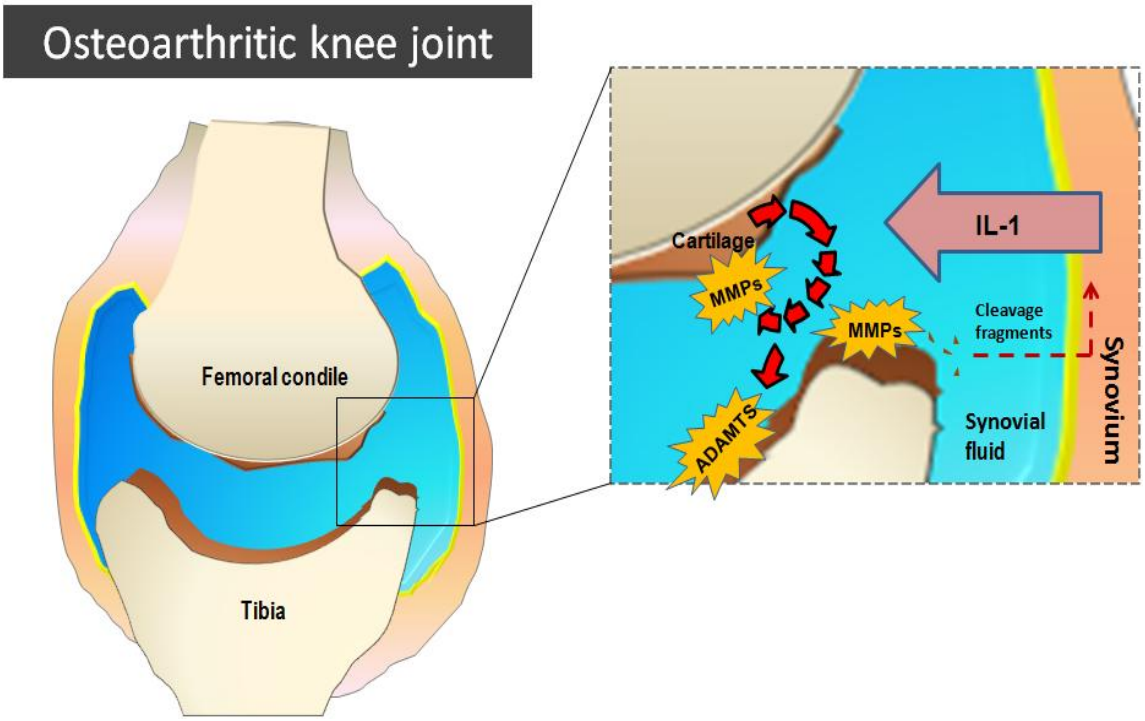

Figure 2. Cartilage tissue breakdown during osteoarthritis. Overuse or injury of the knee joint can cause chronic inflammation of the surrounding tissue including the synovium, which can produce inflammatory cytokines such as IL-1. Inflammatory cytokines trigger the release of proteolytic enzymes such as MMPs and aggrecanases which further degrade the cartilage tissue producing free floating extracellular matrix fragments creating a feedback loop that prolongs inflammation.

FIGURE 3

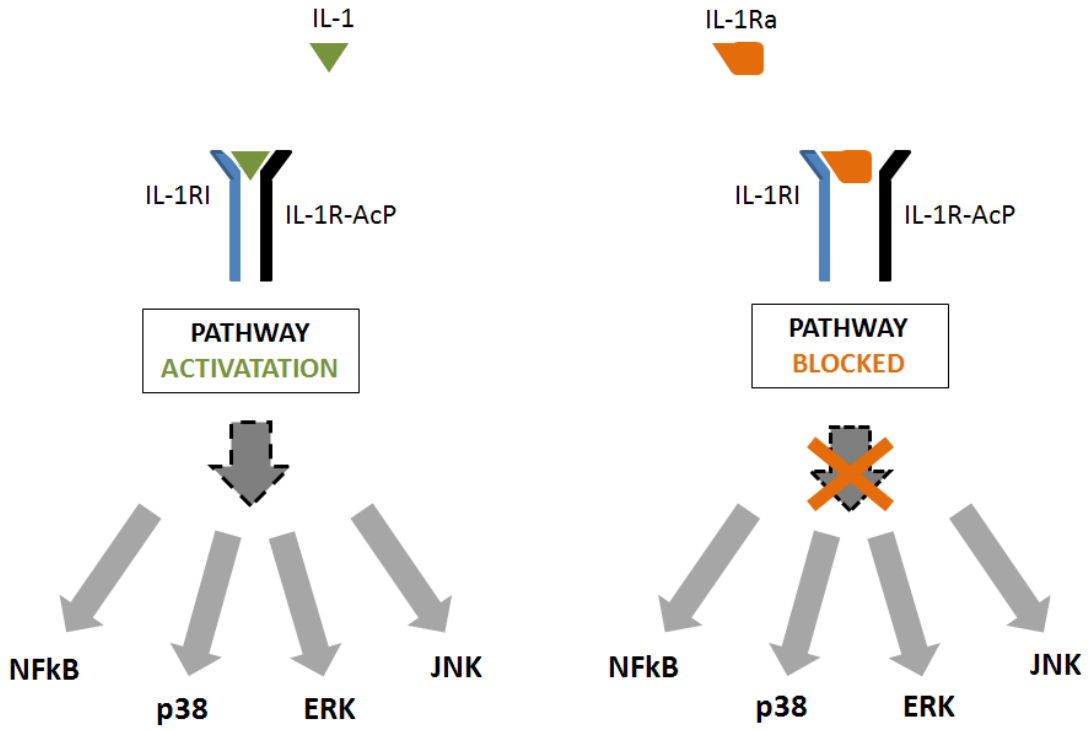

Figure 3. IL-1 receptor antagonist mediated inhibition of IL-1 pathway signaling. IL-1 receptor I (IL-1RI) is a receptor tyrosine kinase that is activated when IL-1 binds and induces the colocalization of IL-1 receptor accessory protein (IL-1R-AcP). IL-1RI and IL-1R-AcP cross phosphorylate each other initiating IL-1 pathway signaling, which eventually activates NFkB and the MAP kinases, as shown on the left. IL-1 receptor antagonist (IL-1Ra) is a competitive inhibitor of IL-1RI ligands. This anti-inflammatory cytokine binds IL-1RI preventing colocalization of IL-1R-AcP and thereby blocking pathway signaling, as shown on the right.

FIGURE 4

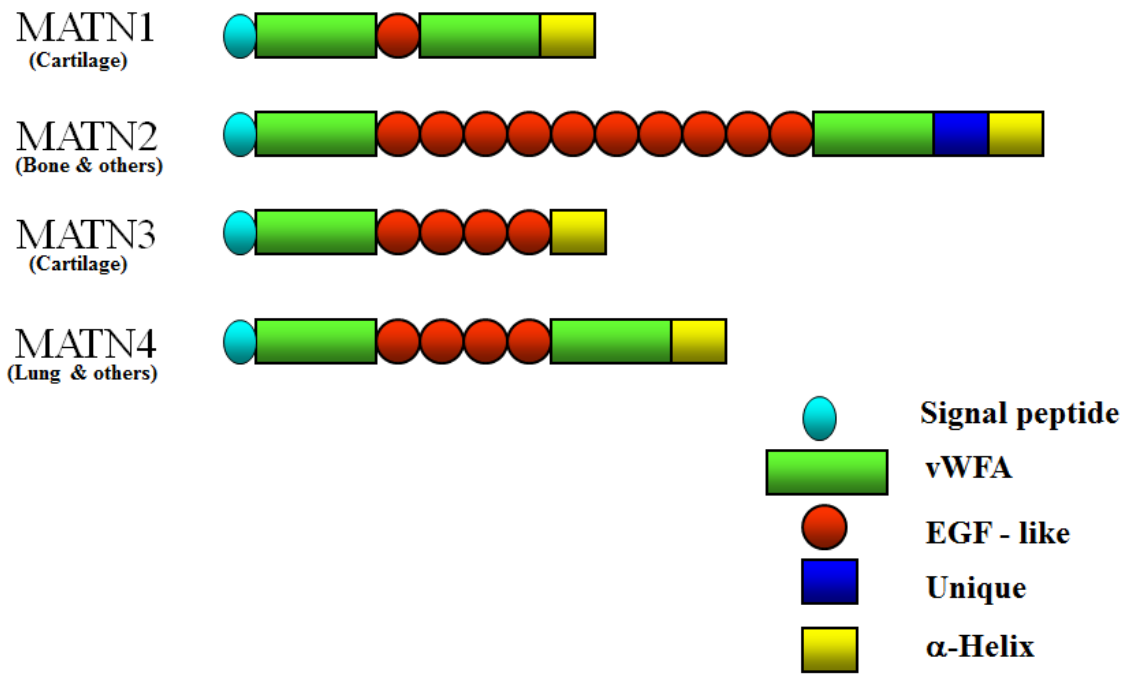

Figure 4. Protein domains of each member of the matrilin family and their respective positions. Each member of the matrilin (MATN) family of extracellular matrix (ECM) proteins consist of a signal peptide, von willebrand factor A domain(s) (vWFA), epidermal growth factor-like domain(s) (EGF-like), and a single alpha-helical oligomerization domain. MATN1 and MATN3 are specifically found in cartilage tissue, whereas MATN2 and MATN4 are found in a wider distribution of tissues.

FIGURE 5

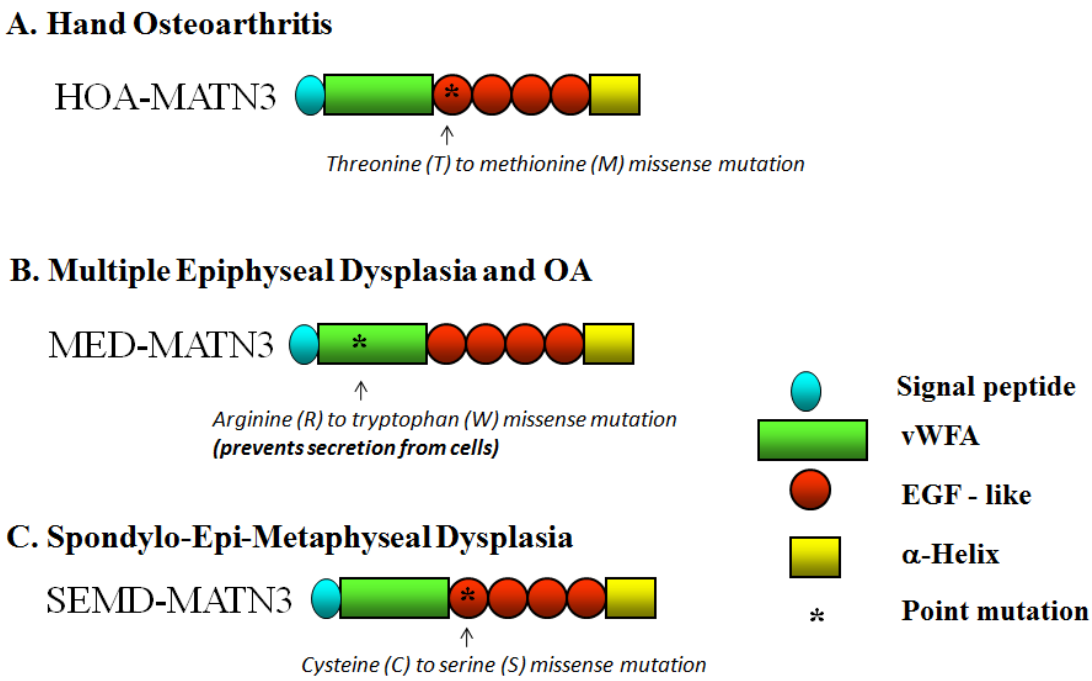

Figure 5. Locations of common matrilin-3 point mutations. A threonine to methionine missense mutation in the first EGF-like domain is associated with the development of hand osteoarthritis (HOA) in patients **(A)**. An arginine to tryptophan mutation in the vWFA domain is associated with multiple-epiphyseal dysplasia (MED), which eventually leads to osteoarthritis (OA) **(B)**. A cysteine to serine missense mutation is associated with spondylo-epi-metaphyseal dysplasia (SEMD) **(C)**.

CHAPTER 2

AIM I

Matrilin-3 Induction of IL-1 Receptor Antagonist Is Required for Up-regulating Collagen II and Aggrecan and Down-regulating ADAMTS-5 Gene Expression

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Matrilin-3 Induction of IL-1 Receptor Antagonist Is Required for Up-regulating Collagen II and Aggrecan and Down-regulating ADAMTS-5 Gene Expression

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Keywords: Matrilin-3, interleukin-1, IL-1Ra, collagen 2, aggrecan, MMP-13, ADAMTS-4, ADAMTS-5, chondrocytes, osteoarthritis.

Abstract

Introduction: Deletion or mutation of the gene encoding the cartilage extracellular matrix (ECM) protein matrilin-3 (MATN3) results in the early onset of osteoarthritis (OA), suggesting chondroprotective properties of MATN3. To understand the mechanisms underlying these properties, we determined the effects of MATN3 protein on the expression of several key anabolic and catabolic genes involved in chondrocyte homeostasis, and the dependence of such regulation on the anti-inflammatory cytokine: IL-1 receptor antagonist (IL-1Ra).

Methods: The effects of recombinant human (rh) MATN3 protein were examined in C28/I2 immortalized human chondrocytes, primary human chondrocytes (PHCs), and primary mouse chondrocytes (PMCs). Messenger RNA levels of IL-1Ra, COL2A1, ACAN, MMP-13, and ADAMTS-4 and -5 were determined using real-time RT-PCR. Knocking down IL-1Ra was achieved by siRNA gene silencing. IL-1Ra protein levels were quantified by ELISA and the Bio-Plex Suspension Array System. COL2A1 protein level was quantified using Western blot analysis.Statistic analysis was done using the two-tailed t-test or one-way ANOVA.

Results: rhMATN3 protein induced gene expression of IL-1Ra in C28/I2 cells, PHCs, and PMCs in a dose- and time-dependent manner. Treatment of C28/I2 cells and PHCs with MATN3 protein stimulated gene expression of COL2A1 and ACAN. Conversely, mRNA levels of COL2A1 and ACAN were decreased in MATN3 KO mice. MATN3 protein treatment inhibited IL-1β-induced MMP-13,

ADAMTS-4 and ADAMTS-5 in C28/I2 cells and PHCs. Knocking down IL-1Ra abolished the MATN3-mediated stimulation of COL2A1 and ACAN and inhibition of ADAMTS-5, but had no effect on MATN3 inhibition of MMP-13 mRNA.

Conclusion: Our findings point to a novel regulatory role of MATN3 in cartilage homeostasis due to its capacity to induce IL-1Ra, to up-regulate gene expression of the major cartilage matrix components, and to down-regulate the expression of OA-associated matrix-degrading proteinases in chondrocytes. The chondroprotective properties of endogenous MATN3 depend partly on its induction of IL-1Ra. Our findings raise a possibility to use rhMATN3 protein for anti-inflammatory and chondroprotective therapy.

Introduction

Matrilin-3 (MATN3) is one of the four members of the matrilin family of noncollagenous oligomeric ECM proteins [1-4]. As the smallest member of this family, it contains a single Von Willebrand Factor A (vWFA) domain, four epidermal growth factor-like (EGF) domains, and an alpha-helical oligomerization domain, which allows it to form oligomers with itself or other matrilin molecules [5]. As an extracellular matrix (ECM) protein, MATN3 was thought to play a major structural role in forming a filamentous matrix network by interacting with collagen fibrils, multiple proteoglycans, and other glycoproteins [5]. Mutations in human MATN3 are associated with a variety of skeletal diseases including multiple epiphyseal dysplasia (MED), spondylo-epi-metaphyseal dysplasia (SEMD), and osteoarthritis (OA) [6-9], underscoring its importance in cartilage development and homeostasis. Deletion of the MATN3 gene in mice results in no gross skeletal deformity at birth; it does, however, cause acceleration of cartilage degeneration during aging [10]. Furthermore, MATN3 gene expression is increased in articular cartilage tissues from OA patients [11].

OA is characterized as a disregulation of cartilage homeostasis due to excessive upregulation of catabolic factors and the inability of the chondrocytes to adequately repair the degraded matrix, resulting ultimately in degeneration of the major cartilage ECM components such as type II collagen fibrils and aggrecan [12-15]. ECM catabolism is largely mediated by the matrix metalloproteinase (MMP) family of collagenases, including MMP-13, and by the ADAMTS family of aggrecanases (ADAMTS-4 and -5) [14-21]. They are often

expressed in chondrocytes in response to major inflammatory cytokines such as IL-1β produced by the synovium and other joint tissues [12]. Furthermore, there is a correlation between the increased levels of these catabolic enzymes and inflammatory mediators such as prostaglandins, nitric oxide (NO), and pro-inflammatory cytokines such as IL-1β and TNF-α in synovial fluids and joint tissue. Previous studies have Implicated IL-1β as one of the major inflammatory cytokines associated with cartilage damage [12, 22 - 25] due to its ability to induce or up-regulate the expression of proteinases, including MMPs, plasminogen activator, and aggrecananases [25 – 30], and to downregulate the expression of endogenous proteinase inhibitors (e.g. certain TIMPs) [22, 31] and cartilage matrix components such as COL2A1 and ACAN [32 – 34]. IL-1 stimulates production of pro-inflammatory factors including prostaglandins, leukotrienes and itself [12, 35, 36]. Importantly, IL-1, IL-1 receptor, and MMPs are expressed by chondrocytes in OA cartilage and can be immunolocalized to the same regions [37 – 40]. Inhibition of the IL-1β pathway presents a promising means of preventing cartilage degradation during OA pathogenesis. One of the major endogenous inhibitors of the IL-1 pathway is IL-1 receptor antagonist (IL-1Ra) [41 – 43].

In this study we aimed to test whether MATN3 can positively regulate cartilage homeostasis genes, including those downstream of IL-1, in a manner that can help explain its chondroprotective function. Here we report for the first time several novel regulatory functions of MATN3 including induction of IL-1Ra, stimulation of COL2A1 and ACAN expression, and inhibition of MMP-13 and

ADAMTS-4 and -5 expression. We also tested whether these novel regulatory properties of MATN3 depend on its induction of IL-1Ra.

Materials and Methods

MATN3 knock-out animals

For the purpose of comparing Col2a1 and Acan expression between wild-type and MATN3 KO mice of the same C57BL/6J genetic background, mRNA was extracted from the whole hind limbs of these mice at embryonic day 18.5. Generation of MATN3 KO mice have previously been described [10]. An in-frame stop codon was introduced into the second exon of their MATN3 gene via homologous recombination during the embryonic stem cell stage rendering these mice functionally MATN3 null animals. All experiments were performed with the approval of the Rhode Island Hospital Institutional Animal Care and Use Committee (IACUC #0231-11).

Isolation of primary chondrocytes from human and mouse cartilage

Primary mouse chondrocytes (PMCs) were isolated from the rib cages of 6-day-old mice. Primary human chondrocytes (PHCs) were isolated from normal-looking areas of knee articular cartilage obtained from 5 patients undergoing total joint replacement surgery. Cartilage samples were individually handled and processed separately to isolate chondrocytes. Each cartilage sample was washed twice with sterile PBS within 2 hours of tissue collection and diced into small fragments. Cartilage fragments were digested in 5.0 mL of Pronase

(Roche, Indianapolis, IN, USA) in HBSS at a concentration of 2.0 mg/mL for 30 minutes at 37 °C under shaking conditions. The digestion solution was removed and cartilage was washed twice with DMEM medium (Life Technologies, Grand Island, NY, USA). After a second wash, DMEM was removed and replaced with 5.0 mL of Type IA Crude Bacterial Collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 1.0 mg/mL for 8 hours at 37 °C under shaking conditions. Collagenase enzyme reaction was stopped by adding 5.0 mL of DMEM medium containing 10% FBS (Life Technologies, Grand Island, NY, USA) into the digestion mix. Solution was filtered using a 100 µm nylon cell strainer (BD, Franklin Lakes, NJ, USA) to remove clumps followed by centrifugation at 1500 rpm to pellet the successfully isolated chondrocytes. Pellet was washed twice with DMEM medium and cells were counted using a hemocytometer. Finally, chondrocytes were plated at high density (4.0×10^6 cells) in 60 mm cell culture dishes using DMEM medium supplemented with 10% FBS and 0.2% Streptomycin (Life Technologies, Grand Island, NY, USA). All studies involving patient tissue samples were conducted in accordance with the Institutional Review Board (IRB) of Rhode Island Hospital. The need to obtain patient consent for the collection of cartilage tissue after surgery was waived by the Rhode Island Hospital IRB.

Chondrocyte cell culture studies

Cell culture experiments were conducted using PMCs, PHCs, and C28/I2 immortalized human chondrocytes and involved first plating cells in 6-well culture plates at 200,000 viable cells per well in 1:1 DMEM/F-12 media (Life

Technologies, Grand Island, NY, USA) supplemented with 10% FBS. After 48 hours, the culture medium was replaced with 2.0 mL of serum-free 1:1 DMEM/F-12 and incubated for 5 hours prior to treatment with recombinant human (rh) MATN3 protein (100 ng/mL or 200 ng/mL) (R&D Systems, Minneapolis, MN) and/or rhIL-1β (5.0 ng/mL) (PeproTech, Rocky Hill, NJ) for 8 – 36 hours, unless otherwise stated. To optimize the induction of catabolic proteases (i.e. MMP-13, ADAMTS family) by IL-1 in both primary and immortalized chondrocytes, we used serum free media for the duration of the treatment period as done by a previous study [44].

Small interfering RNA-based silencing of IL-1Ra in PHCs

PHCs were transfected for 48 hours at approximately 40 – 50% cell confluency with a small interfering RNA (IL1RN ON-TARGETplus siRNA) (Dharmacon, Chicago, IL, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The siRNA targets and suppresses the mRNA and protein expression of all four endogenous isoforms of human IL-1Ra. A non-silencing siRNA (Allstars Negative Control siRNA) (Qiagen, Valencia, CA, USA) was used as control. PHCs were plated at 200,000 viable cells per well in 6-well culture plates 24 hours prior to siRNA transfection procedure. For each well, 40 pmol of siRNA and 2.0 ul of Lipofectamine 2000 were utilized for transfection. The 40 pmol of siRNA was first diluted in 100 ul of Opti-MEM I Reduced Serum Media. Likewise, 2.0 ul of Lipofectamine 2000 was diluted in 100 ul of Opti-MEM I Reduced Serum Media. After 5 minutes at room

temperature, the siRNA and lipid mediator solutions were gently mixed, incubated at room temperature for 20 minutes, and then added to cell culture wells containing 1.0 ml of incomplete DMEM media. Cells were placed in a 37°C incubator and media was changed after 8 hours. PHCs were treated with MATN3 and/or IL-1β 48 hours post transfection for subsequent cell culture experiments.

Gene expression analysis

Total RNA was isolated from PMCs, PHCs, C28/I2 cells and the hind limbs of embryonic day 18.5 wild-type and MATN3 KO mice on the C57BL/6J genetic background [10] using the RNAqueous Kit (Ambion, Austin, TX, USA) according to manufacturer's instructions. Gene expression analysis was conducted by real-time quantitative polymerase chain reaction (RT-qPCR) with the DNA Engine Opticon 2 (Bio-Rad, Hercules, CA, USA) using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA). For RT-qPCR, 0.5 ug of RNA was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The cDNA of each sample was subjected to RT-qPCR using species-specific primer pairs for genes encoding IL-1Ra, type II collagen, aggrecan, MMP-13, and soluble (s)IL-1Ra. Exact primer sequences can be found in Table 1. Relative transcript levels were calculated using the delta delta Ct ($\Delta\Delta$Ct) method, normalized to rRNA 18S expression according to the following equation: $X = 2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = (Ct_{Exp} - Ct_{18S}) - (Ct_{Ctl} - Ct_{18S})$ and X = Relative transcript; Ct_{Ctl} = Ct of control group.

Protein analysis

The Human IL-1ra/IL-1F3 Immunoassay (R&D Systems, Minneapolis, MN) and the MMP-13 Human ELISA Kit (Abcam, Cambridge, MA) were used to quantify IL-1Ra and MMP-13 protein levels, respectively, in PHCs 24 hours following treatment with MATN3 and/or IL-1β according to the manufacturer's instructions. PHCs were seeded at a cell density of 100,000 cells/well in 12-well cell culture plates. After 24 hours, media was changed to be serum-free and each group was treated with MATN3 and/or IL-1β as appropriate. Conditioned media was collected 24 hours later, spun down to remove any cell debris and immediately frozen down at -80°C until assays were ready to be performed. The minimum detectable level of IL-1Ra and MMP-13 by these assays were 30 pg/ml and 6.0 pg/ml, respectively. The Bio-Plex Suspension Array System (Bio-Rad, Hercules, CA, USA) was used to measure soluble IL-1Ra protein levels in cell culture medium of PHCs transiently transfected with either a siRNA against IL-1Ra or a non-silencing scrambled control siRNA. Transiently transfected PHCs were collected 36 hours following treatment with MATN3 and/or IL-1β. These samples were analyzed using Bio-Plex Pro Human Cytokine Assay in duplicate per each biological replicate (two per treatment group) according to the manufacturer's instructions. Protein quantification of type II collagen was conducted via western blot analysis using standard protocols. Prior to Western blot analysis of type II collagen protein, primary human chondrocytes transfected with either the IL-1Ra silencing siRNA or the non-silencing scrambled control siRNA, were seeded and cultured for 48 hours in DMEM containing 10% FBS in

the presence and absence of rhMATN3 (200 ng/mL) and/or IL-1β (5.0 ng/mL). Cell culture pellets were resuspended in 50 uL of RIPA buffer (Cell Signaling Technology, Boston, MA, USA) containing protease inhibitors. Protein concentrations were determined using a Bradford Assay (Bio-Rad, Herculese, CA, USA), according to the manufacturer's instructions, and a NanoDrop 2000c (Thermo Fisher Scientific, Rockford, IL, USA). Western blot analysis was performed using standard protocols. A previously characterized mAb against collagen II [45, 46] (NeoMarkers, Fremont, CA, USA) was used as the primary antibody. Beta-actin was used as a loading control for normalization. Imaging was done using the Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE, USA). Western band intensities was quantified using ImageJ software program (NIH, Bethesda, MD).

Statistical analysis

Mean values were calculated and presented with error bars representing ±SDM (one standard deviation of the mean). Two-tailed t-tests were used to analyze data represented in Figure 1B and Figure 2D. For all else, statistical analysis was done using a one-way ANOVA followed by post hoc test analysis. Statistical significance was considered at $P < 0.05$ for all analyses.

Results

Matrilin-3 induces gene expression of interleukin-1 receptor antagonist

IL-1Ra is a potent endogenous inhibitor of the IL-1 pathway [37, 38]. To determine whether MATN3 affects IL-1Ra synthesis, we treated immortalized human C28/I2 chondrocytes with rhMATN3 protein. MATN3 induced mRNA expression of IL-1Ra in a dose-dependent manner (Figure 1A). We also observed an increase of IL-1Ra mRNA levels in primary mouse chondrocytes (PMCs) treated with rhMATN3 protein (Figure 1B). RhMATN3 protein induced IL-1Ra mRNA in primary human chondrocytes (PHCs) in the presence or absence of IL-1□ (Figure 1C). Treating PHCs with MATN3 , in the presence of IL-1β, significantly enhanced IL-1Ra protein concentration in culture medium compared to that of cells treated with IL-1β alone (Figure 1D). The kinetics of IL-1Ra mRNA induction by MATN3 in C28/I2 cells and PHCs was also analyzed (Supplementary Figure 1). The induction of IL-1Ra mRNA persisted during a 24-hour treatment period with more pronounced induction in the shorter incubation period. Thus, MATN3 induces the mRNA and protein expression of IL-1Ra in chondrocytes.

Matrilin-3 stimulates expression of COL2A1 and ACAN

To investigate whether MATN3 stimulates type II collagen gene (COL2A1) expression in chondrocytes, we treated C28/I2 cells (Figure 2A) and PHCs (Figure 2B) with rhMATN3 protein for 8 hours. We observed a significant induction of COL2A1 expression in these chondrocytes. MATN3 induction of COL2A1 was also observed after 24 hours treatment (Supplementary Figure 2). While treatment with IL-1β significantly decreased COL2A1 mRNA levels in both

C28/I2 cells and PHCs, treatment with MATN3 at the same time reversed this decrease (Figure 2A, 2B). MATN3 treatment also increased aggrecan gene (ACAN) expression by PHCs in the presence of IL-1β (Figure 2C).

To determine whether the lack of MATN3 affects the expression of COL2A1 and ACAN in chondrocytes, we analyzed RNA isolated from the limbs of MATN3 KO and wild type mice. The MATN3 KO mice exhibited 50% reduction of COL2A1 and ACAN mRNA levels compared to their wild-type littermates (Figure 2D) indicating that the absence of MATN3 suppresses the expression of these two genes.

Matrilin-3 inhibits gene expression of MMP-13, ADAMTS-4 and ADAMTS-5

We next examined whether MATN3 could inhibit the expression of IL-1β-induced protease genes. In C28/I2 cells, IL-1β treatment increased MMP-13 mRNA levels and MATN3 treatment decreased MMP-13 expression in both the presence and absence of IL-1β (Figure 3A). IL-1β treatment also increased MMP-13 gene expression by PHCs and elevated MMP-13 mRNA levels and protein concentrations in PHC conditioned media while treatment with MATN3 abrogated this increase significantly (Figure 3B, 3C). Likewise, the IL-1β-induced ADAMTS-4 and -5 gene expression was inhibited by MATN3 in C28/I2 cells (Figure 3D), and in PHCs (Figure 3E).

Interleukin-1 receptor antagonist mRNA and soluble protein levels are diminished by IL1Ra siRNA

Previous studies have shown that IL-1Ra antagonizes IL-1β stimulation of catabolic gene expression and its inhibition of anabolic gene expression. We hypothesize that the chondroprotective properties of MATN3 is dependent on its upregulation of IL-1Ra. To test whether MATN3 acts through IL-1Ra to regulate the expression of anabolic and OA associated catabolic genes, we first knocked down all isoforms of IL-1Ra using a small interfering RNA (siRNA). Since the endogenous soluble IL-1Ra protein levels present in C28/I2 cell supernatants were too low to accurately quantify, all knock-down experiments were conducted in PHCs. The siRNA transfection successfully knocked down both IL-1Ra mRNA (Figure 4A, 4B) and protein expression (Figure 4C) in PHCs both in the presence and absence of IL-1β.

Matrilin-3 stimulation of COL2A1 and ACAN depends on interleukin-1 receptor antagonist

We then determined the effect of MATN3 on COL2A1 expression in IL-1Ra knocked down PHCs. In the control cells, which were transfected with a scrambled siRNA construct, MATN3 treatment rescued the IL-1β induced downregulation of COL2A1 mRNA (Figure 5A, left). COL2A1 protein levels were also enhanced by MATN3 treatment in both the absence and presence of IL-1β (Figure 5B). In contrast, MATN3 was incapable of enhancing COL2A1 mRNA (Figure 5A, right) or protein levels (Figure 5B) in cells transfected with the IL-1Ra siRNA. Similarly, MATN3 reduced the extent of IL-1β-induced downregulation of ACAN mRNA expression in cells transfected with the scrambled siRNA construct

(Figure 5C, left) but not in cells transfected with the IL-1Ra siRNA (Figure 5C, right).

Matrilin-3 inhibition of ADAMTS-5, but not of MMP-13, depends on interleukin-1 receptor antagonist

To test whether MATN3 inhibition of matrix proteases is dependent on IL-1Ra, we quantified their expression levels in the IL-1Ra knock down chondrocytes. Knocking down IL-1Ra chondrocytes significantly increased the basal level of ADAMTS-5 expression (Figure 6A, compare conditions in the absence of MATN3 or IL-1β). The presence of MATN3 significantly inhibited the upregulation of ADAMTS-5 by IL-1β (Figure 6A, left panel, $p \leq 0.05$). However, this inhibition was not significant in IL-1Ra knock-down chondrocytes (Figure 6A, right panel, $p = 0.37$). Thus MATN3 inhibition of ADAMTS-5 is at least partly dependent on IL-1Ra. In contrast, knocking down IL-1Ra did not increase the basal level of MMP-13 gene expression (Figure 6B). In addition, MATN3 inhibition of MMP-13 gene expression was significant in both control and IL-1Ra knock-down cells suggesting that MATN3 inhibition of MMP-13 is not mediated by IL-1Ra.

Discussion

Mutations in human MATN3 are associated with a variety of human cartilage degenerative diseases including chondrodysplasia and osteoarthritis [6 – 9]. These genetic studies strongly suggest that the normal MATN3 gene

product is chondroprotective. Since MATN3 protein is an ECM protein, an accepted hypothesis is that its chondroprotective activity is due to its structural properties which may help to maintain tissue integrity [47]. In this study however, we hypothesized that MATN3 may also be capable of preventing the IL-1β induced disregulation of cartilage homeostasis genes thereby preventing the hallmark of OA development.

Here we demonstrated that rhMATN3 protein induces gene expression of IL-1Ra in three chondrocyte culture models: immortalized human C28/I2 chondrocytes, primary articular human chondrocytes (PHC) and primary mouse (PMCs) chondrocytes. MATN3 increased IL-1Ra mRNA production in the presence and absence of the inflammatory cytokine IL-1β in a dose and time-dependent manner. Furthermore, MATN3 treatment increased the soluble IL-1Ra protein levels in the presence of IL-1β in chondrocytes, as determined by ELISA and Bio-Plex Array analysis.

IL-1Ra, an IL-1α/β protein mimic, is a major endogenous inhibitor of the IL-1β pathway [12, 41 - 43]. It binds IL-1R1, thereby preventing IL-1 downstream signaling. IL-1Ra is potentially chondroprotective, since it inhibits IL-1β, which is a major stress and inflammation cytokine that is closely associated with OA pathogenesis. The concentration of soluble IL-1Ra decreases with increasing grades of articular chondral damage in human patients [48]. Knocking down IL-1Ra results in the early onset of arthritis in multiple mouse genetic backgrounds. IL-1Ra KO mice bred in both BALB/cA and MFIx129 backgrounds developed severe inflammatory arthritis [49]. Conversely, *in vivo* IL-1Ra gene transfer

reduces surgically induced OA severity in rabbits [50]. The level of IL-1Ra present in the rabbit synovium positively correlated with the reduction in articular cartilage lesions.

Because of its chondroprotective properties, it is of paramount importance to study how IL-1Ra gene expression is regulated. IL-1Ra is produced by many cell types including chondrocytes [51]. It is important to recognize that the chondroprotective effects of IL-1Ra during OA are only observable when the protein is consistently present in the arthritic joint. This explains why short-lived drugs based on IL-1Ra (i.e. AnikinRA) [52], which only last a few hours post-intraarticular injection into human patients have limited efficacy in OA treatment [53]. Thus, finding a means of continuously stimulating autologous IL-1Ra production in chondrocytes may provide an effective alternative for sustaining articular cartilage integrity by dampening inflammation under arthritic conditions. We show here for the first time that MATN3 protein is capable of stimulating IL-1Ra expression in chondrocytes. Our findings may also explain a previous report of increased MATN3 expression in OA patients [11] since articular chondrocytes may be attempting to inhibit IL-1 induced joint tissue destruction by increasing MATN3 production as a means of recovery.

To further test the hypothesis that MATN3 is chondroprotective, we examined the effect of MATN3 on the expression of anabolic genes such as COL2A1 and ACAN and catabolic genes such as ADAMTS-4 and -5 and MMP-13 in chondrocytes. Recombinant human MATN3 protein enhanced COL2A1 expression while inhibiting the IL-1β induced down-regulation of COL2A1 and

ACAN. Conversely, the lack of MATN3 resulted in reduced basal expression of these chondrogenic genes in MATN3 KO mice in comparison to wild-type mice of the same genetic background. The levels of both Col2a1 and Acan were reduced by approximately 50% compared to wild-type mice. Thus, MATN3 is necessary to maintain normal expression of these genes in cartilage. It is interesting that MATN3 knockout mice have relatively normal skeletal development [10] despite reduced levels of Col2a1 and Acan as observed here for the first time. This is consistent with the finding that Col2a1 and Acan heterozygous knockout mice have normal skeletal development at birth [54, 55]. However, our data also show that, while IL-1β treatment greatly reduces the levels of COL2A1 and ACAN in chondrocytes, the presence of MATN3 inhibits this further reduction of matrix synthesis by IL-1β. Since the IL-1β levels are often elevated during OA, the lack of MATN3 may result in a more severe phenotype during skeletal aging than during development. Thus, this newly discovered MATN3 property of inhibiting IL-1β may explain why the lack of MATN3 results in accelerated OA during aging despite relatively normal skeletal development in MATN3 KO mice [10].

MATN3 protein not only maintained expression of anabolic genes including COL2A1 and ACAN in our experiments, but also inhibited expression of catabolic genes induced by IL-1β including MMP-13, ADAMTS-4 and -5. This inhibitory effect correlated with the concentration of MATN3 protein in the culture medium. These catabolic genes are closely related to OA pathogenesis with increased gene expression and/or activity [12]. The lack of the ADAMTS-5 gene also protects cartilage degeneration from abnormal mechanical loading in the

mouse joint [20]. Thus, inhibiting gene expression of these OA-associated matrix proteases induced by IL-1β may also contribute to the chondroprotective properties of MATN3.

Since MATN3 induces IL-1Ra expression and inhibits the effects of IL-1β in chondrocytes, we determined whether these two events are dependent. Knocking down IL-1Ra using siRNA abolished both IL-1Ra mRNA and protein levels in chondrocytes. This was also confirmed by the failure of IL-1β treatment to induce IL-1Ra expression in the IL-1Ra knocked-down chondrocytes. The levels of COL2A1 and ACAN mRNA levels were significantly lower in these IL-1Ra knocked-down chondrocytes compared to wild type chondrocytes. Furthermore, treatment with MATN3 protein failed to significantly enhance COL2A1 and ACAN mRNA levels in the presence of IL-1β. Similarly, Western blot analysis indicated that silencing IL-1Ra resulted in an overall reduction of COL2A1 protein level. Silencing IL-1Ra also completely abolished the induction by MATN3 of the COL2A1 gene product, both in the absence and presence of IL-1β. This suggests that the ability of MATN3 to maintain anabolic markers such as COL2A1 and ACAN in chondrocytes depends on the presence of IL-1Ra.

Our study reveals that MATN3 inhibition of IL-1β -stimulated expression of catabolic proteases is mediated by both IL-1Ra dependent and independent pathways. In the IL-1Ra knocked-down cells, ADAMTS-5 mRNA levels were significantly increased in comparison to wild-type chondrocytes, while MMP-13 mRNA levels remained unchanged. Upon IL-1β treatment, MATN3 inhibition of the stimulation of ADAMTS-5 in IL-1Ra knocked-down chondrocytes became

statistically insignificant. In contrast, MATN3 still significantly inhibited the stimulation of MMP-13 by IL-1β in the knocked-down cells as in wild-type chondrocytes. The same was true for ADAMTS-4 gene expression as well (not shown). This indicates that MATN3 inhibition of IL-1β up-regulation of ADAMTS-5 is IL-1Ra dependent, while its inhibition of IL-1β up-regulation of MMP-13 and ADAMTS-4 is not. In addition to IL-1Ra, there are other antagonists in the IL-1β signaling pathway including a decoy receptor IL-1RII, an inhibitory receptor SIGIRR, and a soluble receptor sIL-1RAcP [56, 57]. MATN3 may inhibit IL-1β stimulation of MMP-13 by up-regulating one of these antagonists. Alternatively, MATN3 may also exert its regulatory function by down-regulating agonists in the IL-1 signaling pathway that are independent of IL-1Ra. A limitation of this study involves the use of a relatively small patient pool (total of 3 to 5 patients) for the acquisition of primary human chondrocytes. The conclusion of MATN3 induction of IL-1Ra is however valid in chondrocytes from each patient. Thus, experiments involving the use of primary human chondrocyte cultures accomplished its intended purpose of corroborating the findings observed in the C28/I2 human chondrocyte cell line.

The mechanism by which MATN3 regulates IL-1Ra is currently unknown. Given that IL-1Ra mRNA transcript levels are elevated relatively quickly (within 24 hours of exposure to MATN3 recombinant protein), the stimulation by MATN3 of this anti-inflammatory cytokine may be a direct effect. Cartilage extracellular matrix molecules such as hyaluronan have previously been shown to mediate regulatory functions by direct interaction with cell surface molecules and/or

receptors [58, 59]. It is possible that chondrocytes have an endogenous receptor through which MATN3 signals to mediate its regulatory function(s). It has been shown previously that matrilins may interact with integrins or other plasma membrane receptors in chondrocytes [60]. It remains to be determined whether the regulatory functions of MATN3, as shown here, are mediated by these receptors.

We have shown that MATN3 treatment inhibits OA associated catabolic gene expression. Interestingly, a previous study has shown that treatment of human chondrocytes with very high concentrations of murine recombinant MATN3 protein leads to activation of OA-associated catabolic genes [61]. It is important to note that there are two clear differences between the aforementioned study and this one. The first is that, while mouse recombinant MATN3 was used to treat human primary chondrocytes in the previous study, we used human recombinant MATN3 to treat human cells in this study. The second, and perhaps a major difference, is the concentration of recombinant MATN3 used to treat chondrocytes. Recombinant MATN3 protein was used at 5 to 50 µg/ml concentrations in the previous study, which is 50 to 500 times higher than the concentrations (100 to 200 ng/ml) used in this study. Proteins at concentrations significantly higher than the physiological levels may exert toxic effects on cells thereby triggering production of degenerative proteases. Indeed, chondrocytes treated with type II collagen at the same high concentrations also induced matrix protease expression in the previous study [61]. Thus, the data from the two studies indicate that MATN3 is chondroprotective at 100 to 200

ng/ml concentration range while stimulating catabolic pathways at 5 to 50 µg/ml concentration range, which may reflect supra-physiological levels.

Conclusion

This study presents evidence of several novel regulatory functions of MATN3 including induction of IL-1Ra expression, maintenance of collagen II and aggrecan gene expression, and inhibition of the IL-1β induced gene expression of certain catabolic matrix proteases including ADAMTS-5. These regulatory functions are MATN3 concentration dependent. We further demonstrate that some of these regulatory functions (i.e. enhancement of COL2A1 and ACAN gene expression) are dependent on IL-1Ra, while other regulatory functions are independent (i.e. inhibition of IL-1β induced MMP-13 gene expression). These observations were made in multiple chondrocyte culture models, including primary and immortalized cells, and human and mouse species. It provides a novel mechanism for chondroprotective properties of MATN3, which has been strongly indicated by previous genetic studies in human and mouse. It suggests that MATN3 plays only not a structural role in cartilage ECM, but also a regulatory role in cartilage homeostasis by modulating genes critical for matrix synthesis, degradation, and inflammation. It also raises an intriguing possibility of using MATN3, a cartilage matrix protein, for stimulating endogenous anti-inflammatory and chondroprotective properties in cartilage.

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TABLE 1

Species	Gene	Forward sequence	Reverse sequence
Human	ADAMTS4	5'-CCCCAGACCCCGAAGAGCCA-3'	5'-CCCGCTGCCAGGCACAGAAG-3'
Human	ADAMTS5	5'-GGCCGTGGTGAAGGTGGTGG-3'	5'-GCTGCGTGGAGGCCATCGTC-3'
Human	ACAN	5'-ACCAGACGGGCCTCCCAGAC-3'	5'-TGGCTCTGCCCCAGAGGGAC-3'
Human	COL21A	5'-TGAGGGCGCGGTAGAGACCC-3'	5'-TGCACACAGCTGCCAGCCTC-3'
Human	IL-1Ra (pair 1)*	5'-CCCGTGAAGGAGAGCCCTTCATTTG-3'	5'-ACTTTCACCATCATTTCACAAATGCAG-3'
Human	IL-1Ra (pair 2)**	5'-TGTTCCATTCAGAGACGATCTGCCG-3'	5'-GAGCATGAGGCTCAATGGGTACC-3'
Human	MMP-13	5'-ATGCGGGGTTCCTGATGTGG-3'	5'-GGCCCAGGAGGAAAAGCATG-3'
Murine	Acan	5'-CAGTGCGATGCAGGCTGGCT-3'	5'-CCTCCGGCACTCGTTGGCTG-3'
Murine	Col2a1	5'-CACACTGGTAAGTGGGGCAAGACCG-3'	5'-GGATTGTGTTGTTTCAGGGTTCGGG-3'
Murine	IL-1Ra	5'-ACCCATGGCTTCAGAGGCAGC-3'	5'-GCCCCCGTGGATGCCCAAG-3'

* Primer pair used for IL-1Ra expression analysis in C28/I2 immortalized human chondrocytes and primary human chondrocytes for Figure 1A, 1C and Supplementary Figure 1.

** Primer pair used for IL-1Ra expression analysis in primary human chondrocytes for Figure 4A and 4B.

Table 1. Forward and reverse primer sequences used for Real-Time PCR

FIGURE 1

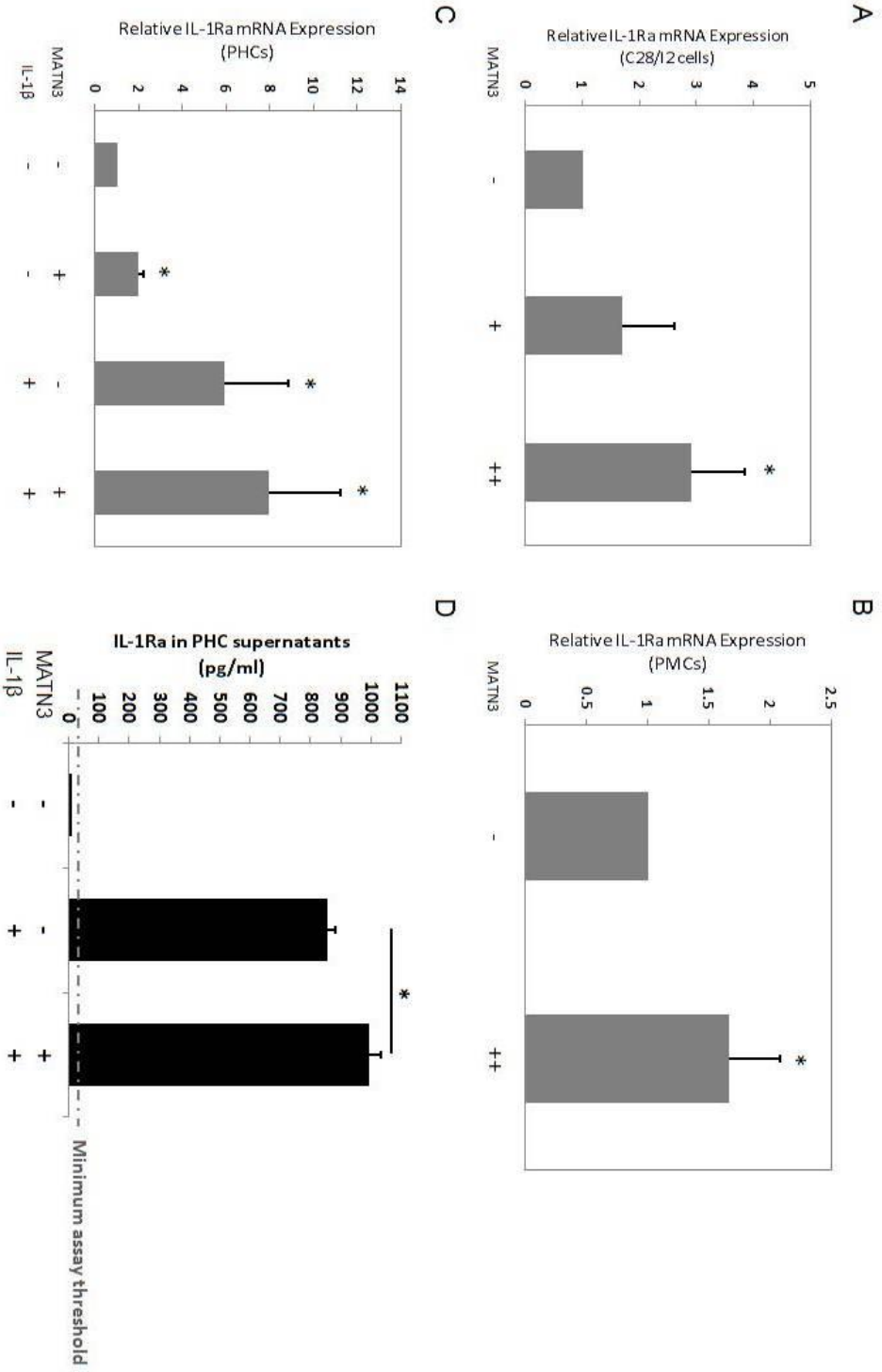

A
Relative IL-1Ra mRNA Expression
(C28/I2 cells)
MATN3
B
Relative IL-1Ra mRNA Expression
(PMCs)
C
Relative IL-1Ra mRNA Expression
(PHCs)
IL-1β
D
IL-1Ra in PHC supernatants
(pg/ml)
Minimum assay threshold

Figure 1. MATN3 enhances IL-1Ra expression by chondrocytes. Recombinant human MATN3 protein enhances the gene expression of IL-1Ra in a dose-dependent manner at 12 hours post cell culture treatment as seen here in C28/I2 cells **(A)**. Recombinant MATN3 also enhances IL-1Ra expression in primary mouse chondrocytes after 24 hours of treatment **(B)**. MATN3 induced IL-1Ra gene expression is evident in PHCs at 24 hours post treatment in both the presence and absence of IL-1β **(C)**. PHCs treated with both MATN3 and IL-1β for 24 hours exhibit significantly higher concentrations of IL-1Ra protein in their cell media, relative to the media of cells treated with IL-1β alone **(D)**. In these experiments, human recombinant MATN3 protein is used at one of two concentrations: lower dose ("+") of 100 ng/ml, or higher dose ("++") of 200 ng/ml. Recombinant human IL-1β protein treatment is always 5.0 ng/ml. Asterisks indicate statistically significant differences of $p \leq 0.05$ from the untreated control group, and hash marks indicate statistically significant differences of $p \leq 0.05$ from the IL-1β only treated group. Data are representative of 3 individual experiments.

FIGURE 2

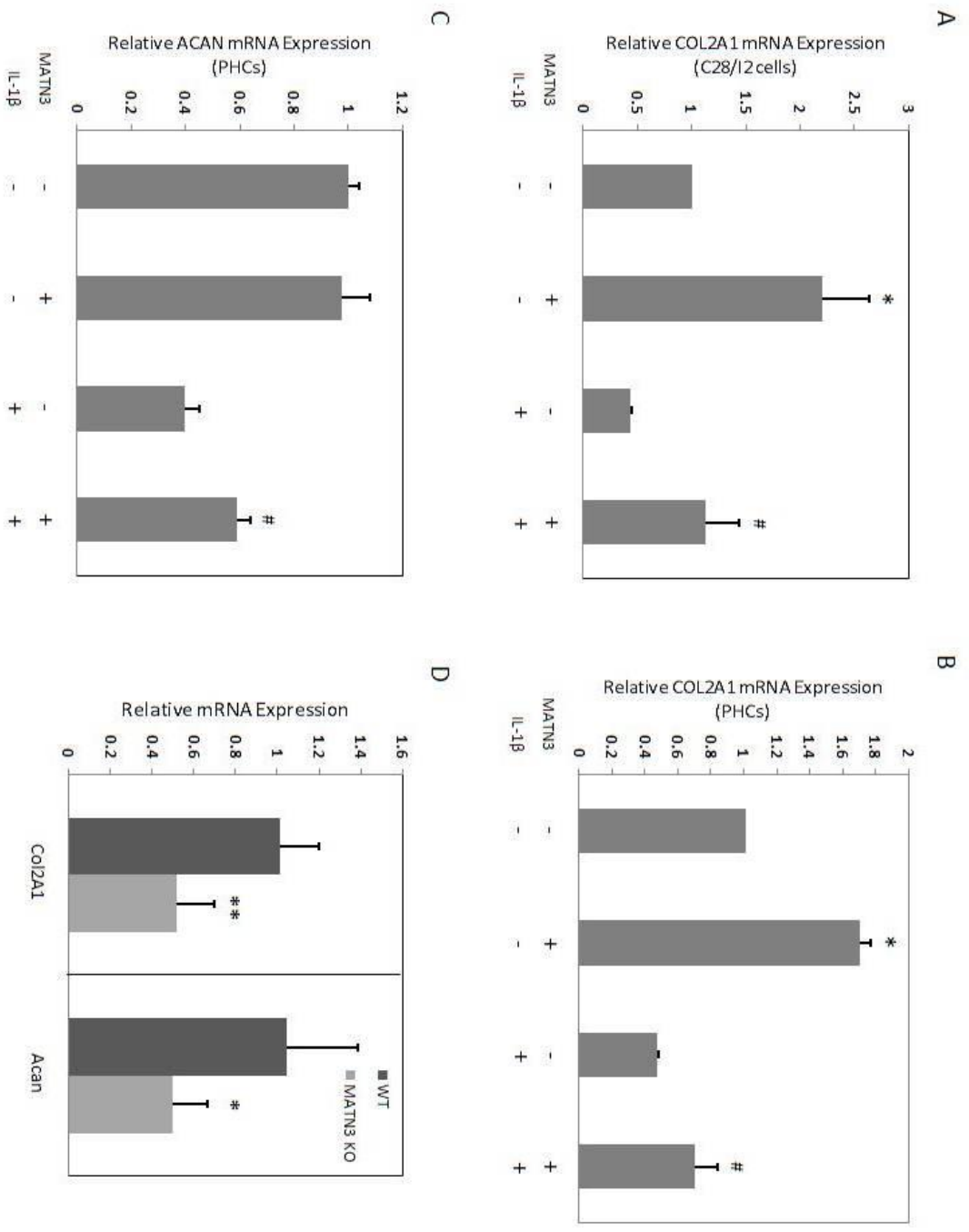

A
Relative COL2A1 mRNA Expression
(C28/I2 cells)
MATN3
IL-1β
B
Relative COL2A1 mRNA Expression
(PHCs)
C
Relative ACAN mRNA Expression
(PHCs)
D
Relative mRNA Expression
Col2A1
Acan
WT
MATN3 KO

Figure 2. MATN3 maintains the expression of key cartilage ECM genes. Recombinant human MATN3 treatment maintains COL2A1 gene expression in the presence and absence of IL-1β in C28/I2 cells **(A)** and PHCs **(B)** at 8 hours. MATN3 also inhibits the IL-1β induced downregulation of ACAN gene expression in PHCs at 36 hours **(C)**. Whole limb mRNA analysis of newborn MATN3 KO mice of the C57BL/6J background reveal lower basal gene expression of Col21a and Acan compared to wild-type mice of the same genetic background **(D)**. For all cell culture experiments, human recombinant MATN3 protein is used at 200 ng/ml. Recombinant human IL-1β protein treatment is always 5.0 ng/ml. Asterisk ($p \leq 0.05$) indicates statistically significant differences from the untreated control group. Hash marks indicates statistically significant differences of $p \leq 0.05$ from the IL-1β only treated group. For cell culture experiments, data are representative of 3 individual experiments. And $n = 5$ for mouse whole limb mRNA studies.

FIGURE 3

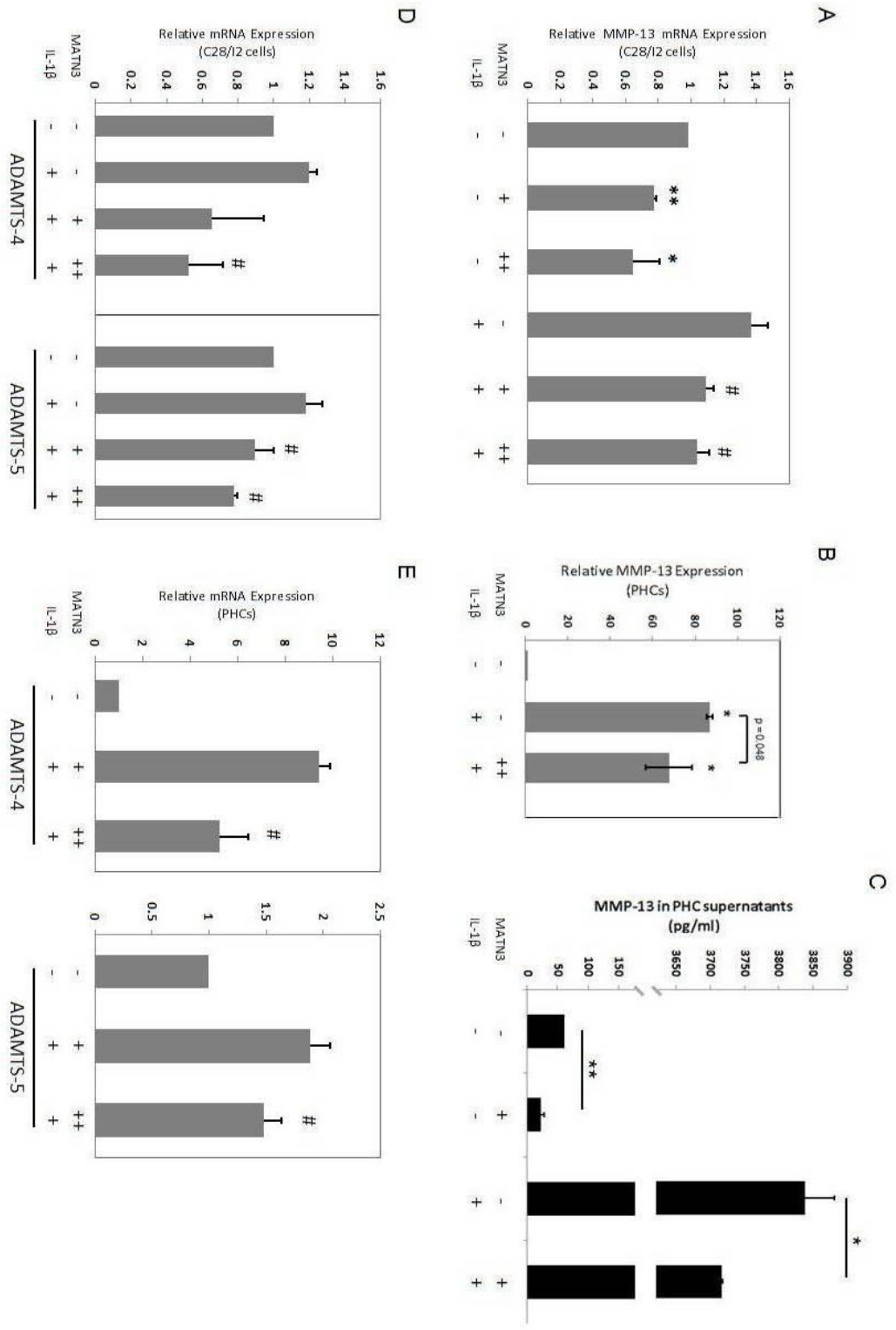

A
Relative MMP-13 mRNA Expression (C28/I2 cells)
MATN3
IL-1β
B
Relative MMP-13 Expression (PHCs)
p=0.048
C
MMP-13 in PHC supernatants (pg/ml)
D
Relative mRNA Expression (C28/I2 cells)
ADAMTS-4
ADAMTS-5
E
Relative mRNA Expression (PHCs)
ADAMTS-4
ADAMTS-5

Figure 3. MATN3 inhibits the expression of several OA associated proteases. Recombinant human MATN3 protein inhibits the gene expression of MMP-13 in a dose-dependent manner in the presence and absence of IL-1β in C28/I2 cells **(A)**. MATN3 inhibitsIL-1β induced MMP-13 mRNA levels **(B)** and protein levels in PHC conditioned media **(C)**. MATN3 inhibits the mRNA expression of OA associated proteases ADAMTS-4 and ADAMTS-5 in C28/I2 cells **(D)** and PHCs **(E)**. MMP-13 mRNA and protein analysis were conducted after MATN3 treatment for 24 hours. In these experiments, human recombinant MATN3 protein is used at one of two concentrations: lower dose ("+") of 100 ng/ml, or higher dose ("++") of 200 ng/ml. Recombinant human IL-1β protein treatment is always 5.0 ng/ml. Single asterisk ($p \leq 0.05$) and double-asterisks ($p \leq 0.01$) indicates statistically significant differences from the untreated control group. Single hash mark ($p \leq 0.05$) and double hash marks ($p \leq 0.01$) indicate statistically significant differences from the IL-1β only treated group. Data are representative of 3 individual experiments.

FIGURE 4

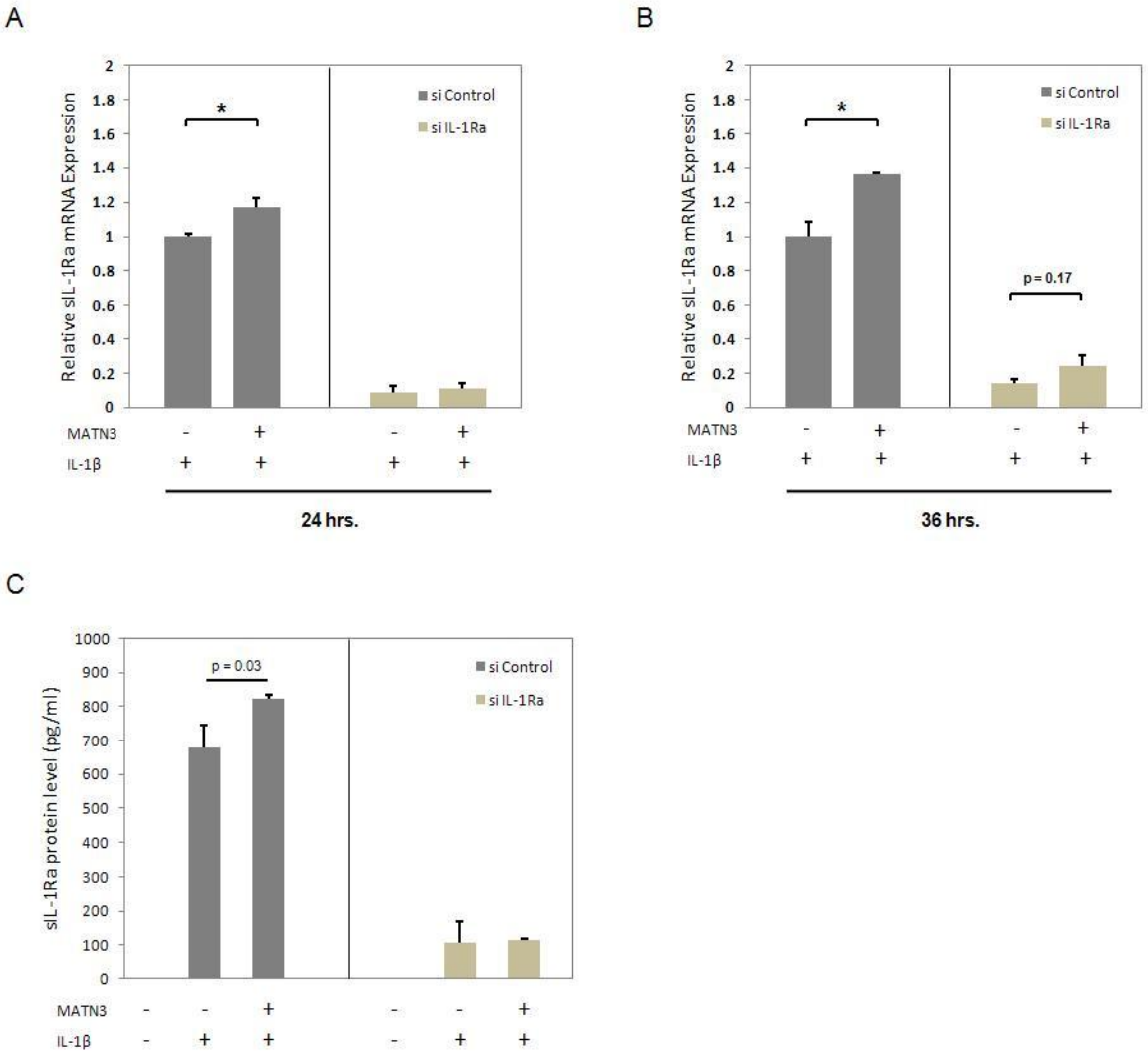

A
Relative sIL-1Ra mRNA Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
si Control
si IL-1Ra
MATN3 - + - +
IL-1β + + + +
24 hrs.
B
Relative sIL-1Ra mRNA Expression
*
p = 0.17
si Control
si IL-1Ra
MATN3 - + - +
IL-1β + + + +
36 hrs.
C
sIL-1Ra protein level (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
p = 0.03
si Control
si IL-1Ra
MATN3 - - + - - +
IL-1β - + + - + +

Figure 4. Small interfering RNA significantly suppresses mRNA and protein levels of IL-1Ra. Knocking down all isoforms of IL-1Ra via IL1RN siRNA treatment reduces the IL-1β induction of this gene as observed at two time points (24, 36 hours) post cell culture treatment **(A, B)**. This also consequently results in the significant reduction of soluble IL-1Ra protein levels in PHC cell supernatants after 24 hours **(C)**. PHCs treated with a nonspecific scrambled siRNA construct is used as the control group. For these experiments, human recombinant MATN3 protein is used at 200 ng/ml. Recombinant human IL-1β protein treatment is always 5.0 ng/ml. Asterisk indicates statistically significant differences of $p \leq 0.05$ between groups. Data are representative of 3 individual experiments.

FIGURE 5

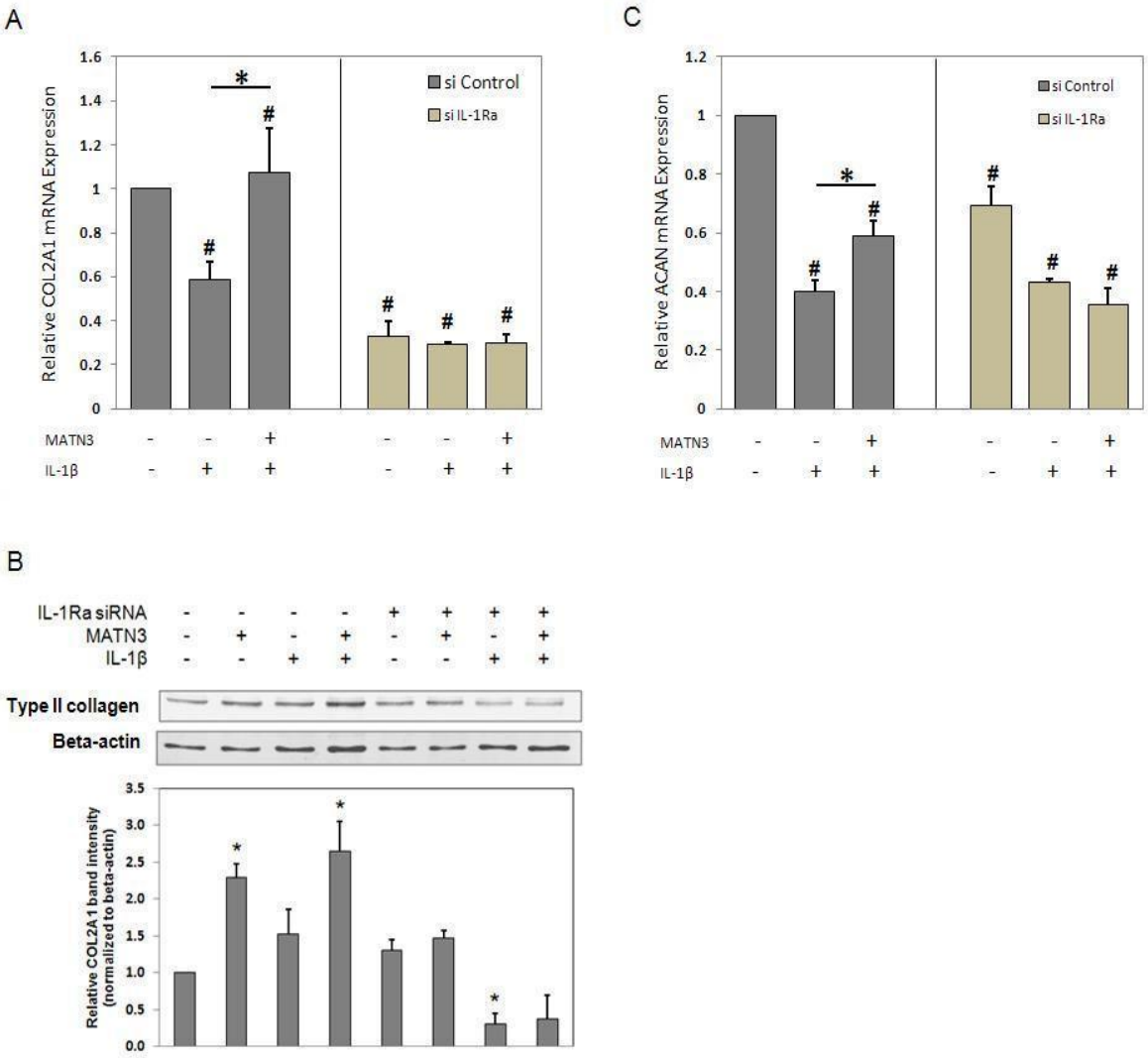

A
Relative COL2A1 mRNA Expression
si Control
si IL-1Ra
MATN3
IL-1β
C
Relative ACAN mRNA Expression
si Control
si IL-1Ra
MATN3
IL-1β
B
IL-1Ra siRNA
MATN3
IL-1β
Type II collagen
Beta-actin
Relative COL2A1 band intensity (normalized to beta-actin)

Figure 5. MATN3's regulation of COL2A1 and ACAN gene expression, as well as COL2A1 protein expression requires IL-1Ra. Knocking down of IL-1Ra abolishes the ability of recombinant human MATN3 protein to maintain COL2A1 gene expression in PHCs that are challenged with IL-1β **(A)**. A significant reduction in the basal gene expression of COL2A1 is also observed in IL-1Ra knock down PHCs. PHCs cultured for 48 hours in media supplemented with recombinant human MATN3 protein (200 ng/ml) exhibit increased COL2A1 protein levels; however, upon suppressing IL-1Ra via siRNA based gene knock down, MATN3's enhancement of collagen is diminished according to western blot analysis **(B)**. Relative quantification of band intensity is the accumulated result of 3 individual experiments (n = 3). Knocking down of IL-1Ra also abolishes MATN3's stimulation of ACAN gene expression **(C)**. The concentration of recombinant human MATN3 protein used was 200 ng/ml and IL-1β was 5.0 ng/ml for all experiments. For A and C, gene expression analysis was conducted 36 hours post exposure to cell culture treatment conditions. Hash marks ($p \leq 0.05$) indicate statistical significance from the untreated group. For A and C, asterisks indicate statistically significant differences of $p \leq 0.05$ between groups; and in B, indicates statistically significant differences of $p \leq 0.05$ from the untreated control group. Individual experiments were done in biological triplicate per patient sample. Data is representative of 5 individual experiments.

FIGURE 6

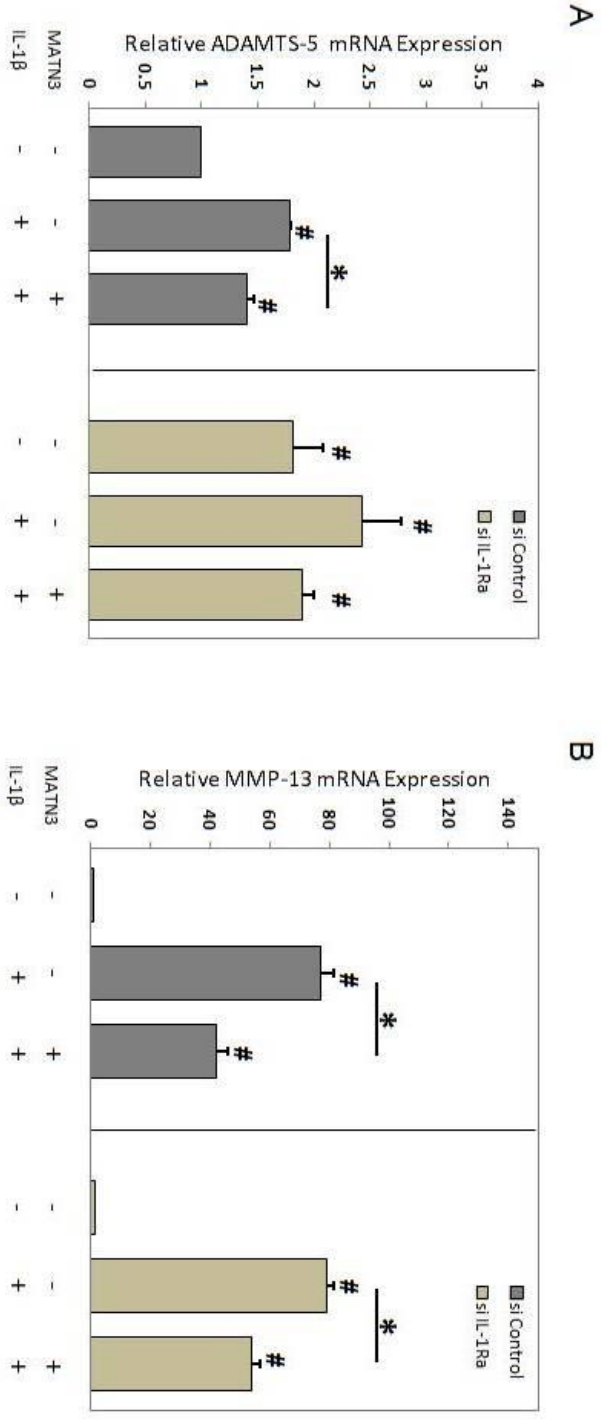
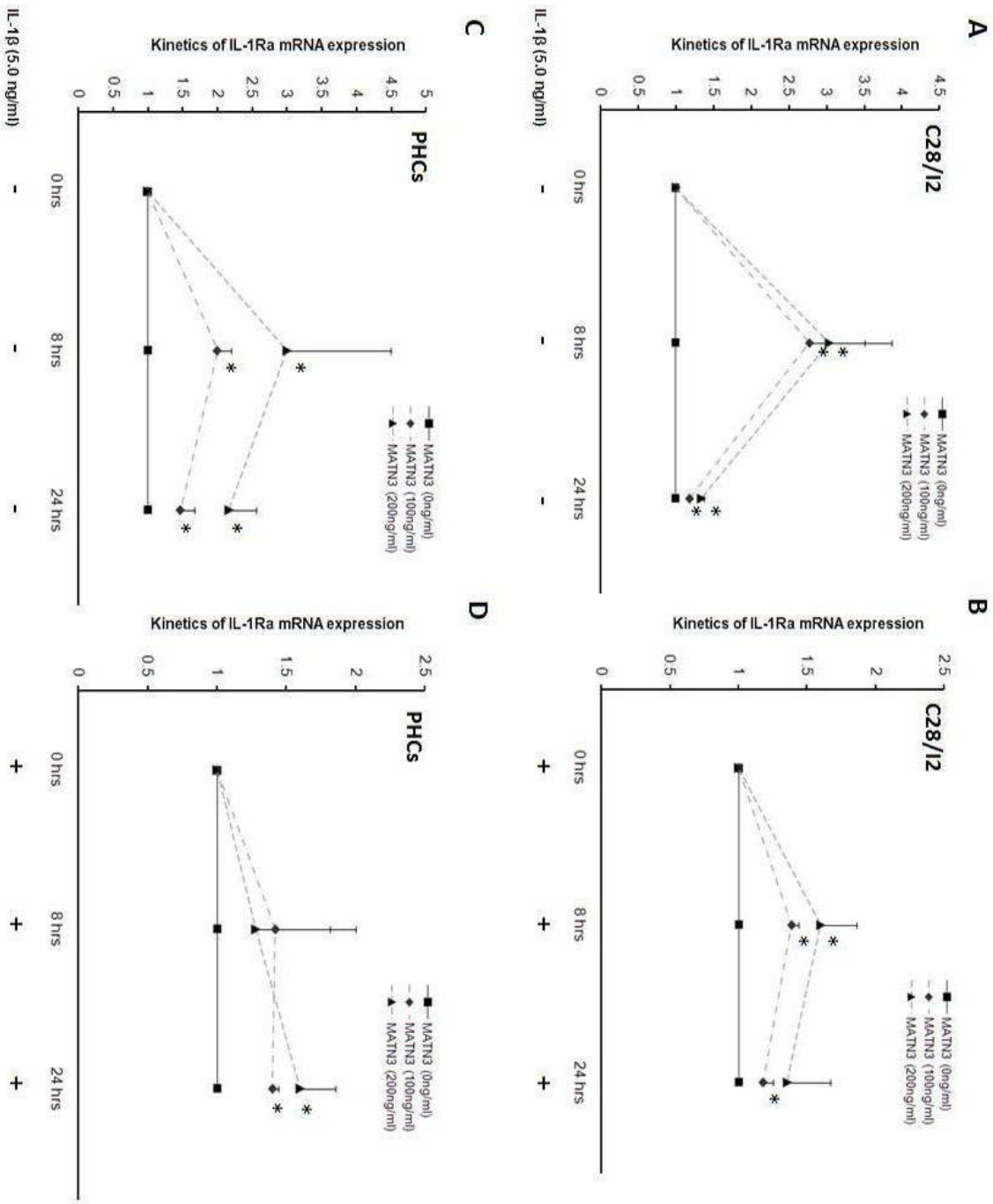

A
Relative ADAMTS-5 mRNA Expression
0
0.5
1
1.5
2
2.5
3
3.5
4
MATN3
IL-1β
si Control
si IL-1Ra
B
Relative MMP-13 mRNA Expression
0
20
40
60
80
100
120
140
MATN3
IL-1β
si Control
si IL-1Ra

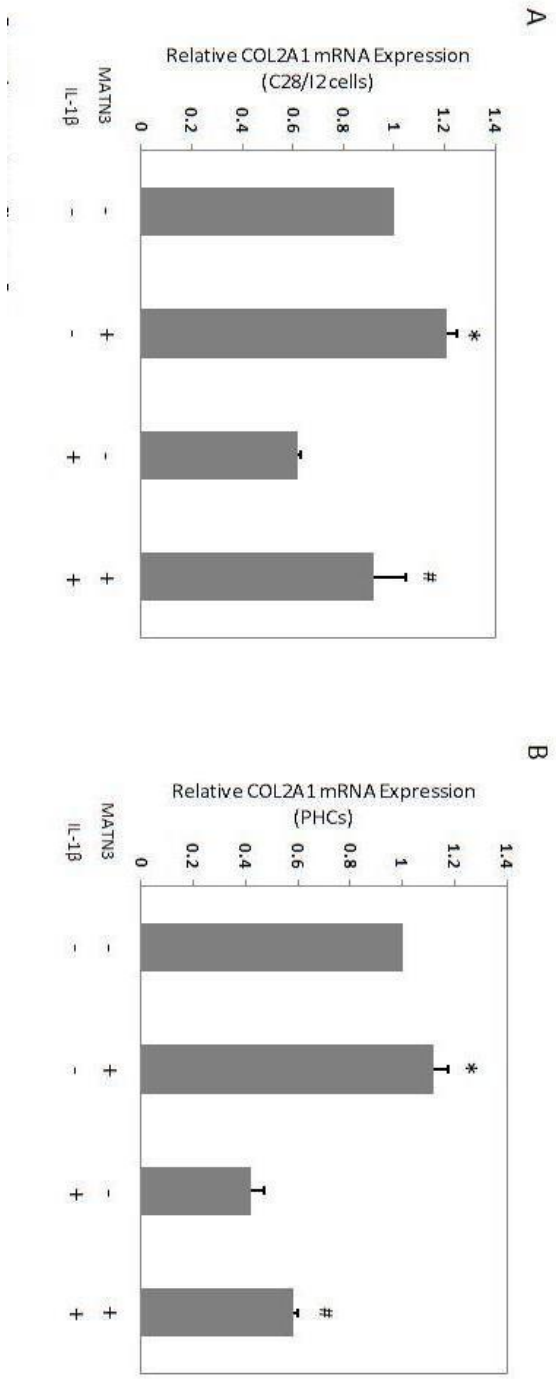
Figure 6. MATN3 inhibition of ADAMTS-5 gene expression require IL-1Ra. Knocking down IL-1Ra in PHCs only partially affects MATN3-induced inhibition of ADAMTS-5 **(A)** and does not seem to significantly affect MMP-13 gene expression **(B)**. The concentration of recombinant human MATN3 protein used was 200 ng/ml and IL-1β was 5.0 ng/ml. Gene expression analysis was conducted 36 hours post exposure to cell culture treatment conditions. Hash marks ($p \leq 0.05$) indicate statistical significance from the untreated group. Asterisks indicate statistically significant differences of $p \leq 0.05$ between groups. Individual experiments were done in biological triplicate per patient sample. Data is representative of 5 individual experiments.

SUPPLEMENTARY FIGURE 1



Supplementary Figure 1. Kinetics of MATN3 induced IL-1Ra gene expression in human chondrocytes. MATN3 stimulation of IL-1Ra gene upregulation by C28/I2 cells and PHCs in the absence **(A, C)** and presence of IL-1β **(B, D)**. Cells were treated with 0, 100 or 200 ng/ml of recombinant human MATN3 protein. IL-1β was used at a concentration of 5.0 ng/ml. Asterisks indicate statistically significant differences of $p \leq 0.05$ relative to the 0 ng/ml treatment group, for each respective time point. Individual experiments were done in biological triplicate.

SUPPLEMENTARY FIGURE 2



Supplementary Figure 2. MATN3 stimulates COL2A1 mRNA levels for at least 24 hours in human chondrocytes. MATN3 induces COL2A1 mRNA levels in C28/I2 cells **(A)** and PHCs **(B)** after 24 hours treatment. Human recombinant MATN3 protein is used at 200 ng/ml and recombinant human IL-1β protein treatment is 5.0 ng/ml. Asterisk ($p \leq 0.05$) indicates statistically significant differences from the untreated control group. Hash marks indicate statistically significant differences of $p \leq 0.05$ from the IL-1β only treated group. Data are representative of 3 individual experiments.

CHAPTER 3

AIM II

Matrilin-3 mutations cause abnormal chondrogenic differentiation of stably transfected ATDC5 murine chondroprogenitors

Compilation of two manuscripts in preparation

Matrilin-3 mutations cause abnormal chondrogenic differentiation of stably transfected ATDC5 murine chondroprogenitors

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Keywords: Matrilin-3, MED, SEMD, HOA, IL-1Ra, chondrogenesis, chondroprogenitors, ATDC5

Abstract

Introduction: Several mutations in the cartilage specific matrilin-3 (MATN3) gene are closely associated with the development of multiple epiphyseal dysplasia (MED), spondyloepimetaphyseal dysplasia (SEMD) and hand osteoarthritis (HOA). This study aims to understand the effects of wild-type MATN3 as well as those of MED, SEMD and HOA MATN3 mutations on chondroprogenitor cells. Furthermore, we look at the effects of silencing the anti-inflammatory cytokine IL-1Ra on the ability of MATN3 to regulate chondrogenesis markers in chondroprogenitor cells.

Methods: ATDC5 murine chondroprogenitors and primary porcine synovial fibroblasts (PSFBs) were stably transfected with pcDNA containing either the wild-type MATN3 gene or constructs carrying MATN3 mutations associated with MED, SEMD or HOA. Immunocytochemistry and Western blot analysis were used to compare wild-type and mutant MATN3 protein levels. Alcian blue and dimethyl-methylene blue was used to measure proteoglycan content. IL-1Ra gene knockdown was achieved through siRNA gene silencing. Real-time PCR was used for quantifying Col2a1, Col10a1, Acan, Ihh, MMP-9, MMP-13, ADAMTS-5, IL-1Ra, Sox9, CD44, CD49e, and CD166 gene expression.

Results: ATDC5 cells transfected with wild-type or mutant MATN3 gene constructs showed increased Alcian blue staining on days 6, 12, and 16 in culture. However, staining was reduced in cells expressing the SEMD-MATN3 mutation compared to wild-type MATN3 transfected cells. PSFBs transfected

with SEMD-MATN3 and HOA-MATN3 exhibited low dimethyl-methylene blue staining. Wild-type MATN3 enhanced Col2a1 and Acan mRNA expression. MED and SEMD-MATN3 point mutations drastically reduced Col2a1 and Acan mRNA expression while enhancing Col10a1 and Ihh expression at most time points. HOA-MATN3 delayed the expression of Col2a1, Acan and induced significantly less IL-1Ra expression than wild-type MATN3. Cells expressing wild-type MATN3 that were treated with IL-1Ra siRNA showed lower levels of Acan, Col2a1 and Sox9 mRNA expression than untreated cells. Recombinant MATN3 protein increased ACAN, COL2A1 and SOX9 mRNA levels in primary human chondroprogenitors.

Conclusion: Wild type and mutant MATN3-expressing chondroprogenitors spontaneously undergo varying degrees of chondrogenesis. The presence of the MED and SEMD mutation significantly alters MATN3-induced chondrogenesis by promoting terminal differentiation and premature hypertrophy, whereas the HOA mutation showed signs of delayed chondrogenesis overall. Furthermore, the HOA mutation hindered the ability of MATN3 to induce IL-1Ra, as well as several chondrogenesis markers that appear to be downstream of this anti-inflammatory cytokine. Knocking down IL-1Ra effectively inhibited the ability of wild-type MATN3 to stimulate key chondrogenesis markers.

Introduction

The severe debilitating effects of diseases involving abnormal cartilage development and/or homeostasis, including chondrodysplasias and osteoarthritis (OA), illustrate the importance of maintaining normal chondrocyte function. During cartilage development, extracellular matrix (ECM) components such as collagens, proteoglycans, and other small non-collagenous matrix proteins have been proposed to help form a microenvironment that is suitable for maintaining the chondrogenic phenotype in differentiating mesenchymal stem cells [1-4]. Matrilin-3 (MATN3) is an extracellular non-collagenous glycoprotein found in skeletal tissues and it is strongly expressed by growth plate chondrocytes during development [5]. It is well integrated into the cartilage ECM, where it aids in maintaining structural integrity of the cartilage tissue. Previously demonstrated previously, MATN3 can directly interact with two collagen species (type II, IX) [6]. In addition to binding collagens, MATN3 also shows biochemical affinity for non-collagenous proteins in the ECM including, decorin and cartilage oligomeric matrix protein (COMP) [7]. Furthermore MATN3 and matrilin-1 (MATN1) are often co-expressed in cartilaginous tissues and can form hetero-oligomers through covalent disulfate linkage between the coiled-coil domains [5, 8-10]. Despite our current understanding of its molecular interactions with other cartilage ECM proteins, the exact biological role of MATN3 remains to be fully elucidated.

Mutations in the MATN3 gene are closely associated with several genetic skeletal disorders including multiple epiphyseal dysplasia (MED),

spondyloepimetephyseal dysplasia (SEMD), and hand osteoarthritis (HOA) [11-17]. In MED patients, there are currently 13 known autosomal dominant missense mutations that have been mapped to exon-2 of MATN3, which cenodes the vWFA protein domain. Three of these mutations were identified in the α-helical region, while the rest were located on one of the six β-strands that comprise the single β-sheet [15, 16, 18-20]. A tryptophan to arginine (R121W) missense mutation in βB-strand of vWFA domain has been identified in approximately one third of all MED patients making it the most common mutation [16, 18]. MED patients vary from normal to short stature and display delayed or irregular epiphyseal ossification often followed by the early onset of OA [11, 20-22]. Likewise, an autosomal recessive missense mutation in which cysteine is substituted by serine (C304S) in the first epidermal growth factor-like domain (EGF-like domain) of MATN3 has been identified in patients with SEMD [17]. SEMD patients are characterized by severe skeletal abnormalities including bow-legged deformity and short stature. In an attempt to further understand the effects of these missense mutations, *in vitro* studies conducted using primary bovine chondrocytes have shown transient expression of murine MATN3 containing MED (R116W) and SEMD (C299S) mutations, which correspond, respectively, to R121W and C304S mutations of the human gene, These mutations resulted in disturbed protein trafficking to Golgi compartments and cellular retention of MATN3 in the endoplasmic reticulum [14]. A MATN3 mutation in which threonine is replaced by metheonine in the first EGF-like domain of MATN3 results in HOA [23]. However, this mutation does not cause

any noticeable abnormalities in the intracellular processing of MATN3 in primary chondrocytes *in vitro* [14].

Although targeted disruption of the MATN3 gene results in normal skeletal development [24, 25] in mice, the MATN3 knockout (KO) mouse model, in which exon 2 has been replaced with a stop codon, exhibits increased hypertrophy and early onset of OA [25]. Furthermore, we have recently shown that treating mature articular chondrocytes with recombinant human MATN3 (rhMATN3) protein enhances the expression of important anabolic chondrocyte marker genes, including type II collagen (COL2A1) and aggrecan (ACAN), in a manner that is dependent on the anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1Ra) [26]. These findings strongly suggest that MATN3's role in cartilage tissue goes beyond simply providing structural stability and that it may also be involved in regulating cartilage development and homeostasis.

To better understand MATN3's potential as a regulator of developmental and homeostasis processes, in this study we first examine the effects of stably transfecting wild-type (WT) and mutant MATN3 constructs into ATDC5 murine chondroprogenitor cells and primary porcine synovial fibroblasts (PSFBs). Here we demonstrate that MATN3 plays a critical role in promoting chondrogenic differentiation and that chondrodysplasia associated MATN3 mutations can severely alter MATN3 induced chondrogenesis by stimulating terminal differentiation and hypertrophy. We also show that MATN3's modulation of chondrogenesis markers in ATDC5 cells and primary human chondroprogenitors is dependent on IL-1Ra. Furthermore, we demonstrate that the HOA associated

(T298M) mutation in the MATN3 gene hinders its capacity to stimulate IL-1Ra as well as several vital chondrogenesis genes.

Materials and methods

The murine teratoma-derived ATDC5 cell line was acquired from RIKEN cell bank, Japan. All cell culture reagents including DMEM:F12, fetal bovine serum, trypsin EDTA solution and Anti V5 antibody were purchased from Invitrogen (Carlsbad, CA, USA). Odyssey blocking buffer and IRDye® 800CW Conjugated Goat anti-Mouse IgG (H+L), Highly Cross Adsorbed were from LI-COR Biosciences (Lincoln, Nebraska, USA). Human Transferrin, Selenite, Trypan blue, Alcian Blue 8GX, and GuHCl were from Sigma-Aldrich (St. Louis, MO, USA).

Cloning and construction of MATN3 cDNA

Wild type MATN3 (WT) and MATN3 with R116W point mutation (MED) gene constructs, were each cloned into expression vectors of pcDNA3.1/V5-His (Invitrogen, Carlsbad, CA) as previously described [27]. Using similar methods, we also generated MATN3 with the C299S point mutation (SEMD) on mouse sequence NM-010770 (Figure 1). Overlapping PCR was performed to create MATN3 cDNA with the SEMD and HOA point mutations using mouse full-length pcDNA3.1MATN3 and Qiagen hotstar Taq (Valencia,CA,USA). Primers are also listed in Figure 1. The size of the 1.4kb MATN3 product was confirmed on a 1% agarose gel. The pcDNA plasmid plus the mutated MATN3 PCR products were

digested with BamHI and Xba I from Roche (Mannheim, Germany). The SEMD and HOA products were ligated into pcDNA and transformed into competent *E.coli* DH5α cells (invitrogen). Colonies grown on ampicillin LB agar were picked and expanded. All plasmids were purified using high pure plasmid isolation kit from Roche to check for the MATN3 inserts with BamHI and Xba I digestion. Furthermore, individual SEMD and MED clones were sequenced with T7 and BGH primers and confirmed to contain the desired nucleotide change in MATN3.

Stable transfection of ATDC5 cells

ATDC5 cells were routinely grown in complete media (DMEM:F12, 5% FBS and 10 µg/ml Transferrin, 3×10^{-8}M sodium selenite) [28], and maintained at 37ºC and 5% CO_2 . Cells were split into 12 well plates and grown until 95% confluent. Transfection was performed according to manufacturer's instruction with Lipofectamine 2000 (Invitrogen). Briefly, 1.0 µg of DNA was mixed with 2.0 µl lipofectamine in serum free Opti-MEM, within 20 minutes at room temperature, the mixture was added to cells and after 24 hours, media was changed. To determine the optimal concentration of Hygomycin, G418 to use for selection; 5.0 µM, 6.0 µM, 7.0 µM or 8.0 µM was added in complete media to select against cells that were not successfully transfected with the plasmid constructs. Untransfected cells were killed by all 4 concentrations after 4 days. Therefore G418 at 5.0 µM was used to treat all transfected ATDC5 cells. After 2 weeks of G418 treatment, 6 individual colonies from each transfected cell line were isolated using sterile loops and cultured to confluency in new 12 well plates with

complete media without G418. All ATDC5 clones containing various MATN3 constructs were subcultured for further gene and protein characterization.

MATN3 gene expression in stably trasnfected ATDC5 clones

Total RNA was extracted from each WT and mutant MATN3 ATDC5 clone grown in 6 well plates for cDNA synthesis, followed by real-time PCR to measure relative MATN3 mRNA levels normalized to ribosomal RNA 18S (detailed procedure described in *RNA Isolation and real-time RT-PCR* section). Untransfected ATDC5 cells were used as control to measure endogenous MATN3 gene expression. As expected, untransfected ATDC5 cells expressed much lower levels of MATN3 than the ATDC5 clones. WT, MED and HOA MATN3 clones exhibited a 3 to 26-fold increase in MATN3 expression while the SEMD clone exhibited a 50 fold increase in MATN3 expression in comparison to untransfected ATDC5 cells. We purposely selected a SEMD clone that carries a high mutant MATN3 copy number to account for the nature of the disease, which is autosomal recessive.

Immunocytochemistry

Each clone was seeded at low density in separate wells of 8 well chamber slides (Nalge Nunc, Rochester, NY, USA). Media was aspirated out and cells were washed 3 times with sterile 1x PBS. Cells were fixed with 70% ethanol, 50 mM glycine, pH 2.0 at -20ºC for 5 minutes, and washed 3 times with cold sterile 1x PBS, pH 7.4. All transfected and untransfected control ATDC5 cells were

incubated with primary mouse anti-V5 (1:200) at 37ºC for 30 minutes, followed by incubation with secondary donkey anti mouse rhodamine (1:200) and Hoechst nuclear dye (1:100) diluted with PBS, 1%BSA, at 37ºC for 30mins. Cells were washed with PBS, 3 times for 5 minutes in between incubations. The detachable chambers were removed from each slide and the slides were mounted with cover slips in 95% glycerol in 1x PBS, 0.5 mg/ml NaAzide (Sigma-Aldrich, St. Louis, MO, USA). Protein expression was visualized with Nikon fluorescence microscope.

SDS-polyacrylamide gel electrophoresis and western blot analysis

Stably transfected ATDC5 cells containing the WT or mutant MATN3 genes as well as the untransfected ATDC5 control cells were grown in monolayer in 6 well culture plates in DMEM:F12+. Cell lysates and supernatants were separately collected for western blot analysis. To concentrate MATN3 proteins from media, 50 µl Ni-Agarose beads (Invitrogen, Carlsbad, CA, USA) were used to isolate His tagged recombinant MATN3 proteins from 1.0 ml of cell supernatants. After 48 hours at 4^{o}C on a shaker, the Ni-agarose beads from all samples were centrifuged and washed 7 times with buffer containing 20 mM potassium phosphate, 500 mM potassium chloride, 10 mM imidazole pH 7.5. Before protein samples were loaded on to 10% SDS PAGE gel, His tagged proteins were dissociated from Ni-Agarose beads by boiling at 95^{o}C for 10 minutes in 30 µl 2x SDS gel loading buffer, then put on ice and centrifuged to separate Ni-Agarose beads. To prepare cell lysates, ATDC5 cells were lysed with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium

deoxycholate, 0.1% SDS) with protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) on ice. The adherent cells were detached with cell scraper and lysates were then further homogenized with 22 gauge needle and syringe. Cell supernatants were collected after centrifugation at 12,000 x g for 10 minutes at 4^{o}C to remove cell debris. Protein concentration was quantified using Quick Start Bradford 1x Dye Reagent (Bio-Rad Hercules, CA, USA) and a Nanodrop 2000c (Thermo Scientific, Wilmington, DE, USA). Under reducing condition, 20 µg total protein from each transfected and untansfected ATDC5 lysate was boiled for 10 minutes in 1x SDS gel loading buffer containing 5% 2-mercaptoethanol, and separated on 10% SDS-PAGE gel. For western blot involving ATDC5 cell lysates, the non reduced or reduced proteins were transferred onto Immobilon-PVDF membrane (Millipore Corp., Bedford, MA, USA) in 25 mM Tris, 192 mM glycine, and 15% methanol. The membranes were blocked in Odyssey® Blocking Buffer for 1 hour and then probed with a mAb against murine V5 (1:5000) (Invitrogen, Carlsbad, CA, USA). IRDye® 800CW Conjugated anti-Mouse (diluted 1:10,000) (LI-COR Biosciences, Lincoln, Nebraska USA) was used as secondary antibody. The mutant and WT MATN3 with V5 tag proteins were visualized in the 800 nm channel using Odyssey infrared image system according to manufacturer's instruction (LI-COR Biosciences, Lincoln, Nebraska USA). Molecular protein marker used is specific for the Odyssey system and is visualized in the 700 nm channel.

Isolation, expansion and characterization of primary human chondroprogenitor cells (CPCs)

Articular cartilage samples were obtained from total joint replacement surgeries conducted at Rhode Island Hospital (RIH) in accordance with the Institutional Review Board (IRB) of RIH. Patient samples were processed separately and were not combined at any stage of the cell isolation procedure. Shortly after tissue samples were collection, they were thoroughly washed with sterile PBS and diced into very small fragments. The fragments were subsequently digested using Pronase (Roche, Indianapolis, IN, USA) in Hank's Balanced Salt Solution (HBSS) at a concentration of 2.0 mg/ml for 30 minutes at 37°C in a shaking water bath. The pronase digestion solution was removed and tissue fragments were washed twice with 5.0 ml of DMEM medium (Life Technologies, Grand Island, NY, USA). The tissue fragments were then further digested with Type IA Crude Bacterial Collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 1.0 mg/ml for 8 hours at 37°C in a shaking water bath. DMEM medium containing 10% fetal bovine serum (FBS) (Life Technologies) was then used to stop the enzymatic reaction. The cell solution was strained through a 100 µm nylon cell strainer (BD, Franklin Lakes, NJ, USA) to remove clumps. Cells were pelleted via centrifugation at 200 x g. Cell pellet were washed twice with DMEM medium and cells were counted using a hemocytometer. Cells were next subjected to differential adhesion using fibronectin. Cells were plated in DMEM at a density of 4000 cells/ml and allowed to attach (for 20 minutes at 37°C) to a 60-mm petri dish that had been coated overnight with 10 mg/ml of fibronectin

(Sigma-Aldrich, St. Louis, MO, USA) in a solution of 0.1 PBS (pH 7.4) containing 1.0 mM MgCl and 1.0 mM $CaCl_2$. After the 20 minutes had passed, media and non adherent cells were aspirated and replaced with fresh DMEM containing 10% FBS with 0.1 mM ascorbic acid, 0.5 mg ml^{-1} L-glucose, 100 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine and 10000 µg ml^{-1} penicillin and 10000 U ml^{-1} streptomycin (DMEM+) [29]. The dish was placed in a 37°C incubator overnight after which adherent cells were counted. Cells were allowed to grow for 12 days under these conditions at which time most had underdone 5 population doublings to form tight clusters of approximately 32 cells (each derived from one cell). Individual cell colonies were then isolated using sterile 150 µl glass cloning cylinders (Sigma-Aldrich, Milwaukee, WI, USA) and transferred to a 12 well cell culture plate. Cells were further expanded in DMEM+ to 95-100% confluency so that there would be enough cells to conduct mRNA (RT-PCR) and/or protein (Western blot) analysis for the purpose of chondroprogenitor cell characterization.

Experimental cell culture conditions

All experiments involving ATDC5 cells were conducted in monolayer cell culture. Each selected WT, MED and SEMD-MATN3 ATDC5 clone and untransfected ATDC5 control were plated in triplicate at 5 x 10^4 cells/well in 12 well plates in DMEM:F12, 5% FBS and 10 µg/ml Transferrin , 3×10^{-8} M sodium selenite). For each experimental time point, duplicate sets of plates were plated. One set was for mRNA extraction and RT-PCR, and the other was used for Alcian blue staining analysis. Experiments involving primary PSFBs were

conducted using a previously described pellet culture system [1]. Transfected and mock-transfected (control) primary PSFBs were detached using trypsin EDTA. 3.0 x 10^5 cells were centrifuged for 10 minutes at 500 x g to obtain cell pellets which were then cultured in 24-well plates for 3, 11 and 18 days in TGF-β1 (10 ng/mL) supplemented High-Glucose DMEM media containing: proline (40 mg/mL), 100 mM dexamethasone, 0.1 mM ascorbic acid 2-phosphate, penicillin (100 U/mL), streptomycin (100 mg/L), insulin (6.25 mg/mL), transferring (6.25 mg/mL), selenous acid (6.25 mg/mL), linoleic acid (5.35 mg/mL) and BSA (1.25 mg/mL). Primary human chondroprogenitor cells were cultured in DMEM containing 10% FBS with 0.1 mM ascorbic acid, 0.5 mg ml^{-1} L-glucose, 100 mM HEPES, 1.0 mM sodium pyruvate, 2.0 mM L-glutamine and 10000 µg ml^{-1} penicillin and 10000 U ml^{-1} streptomycin [29]. Primary human chondrocytes were cultured in DMEM containing 10% FBS and 10,000 µg ml^{-1} penicillin and 10,000 U ml^{-1} streptomycin. Culture media was changed every 2 days.

RNA isolation and real-time RT-PCR

The RNAqueous Kit (Ambion, Austin, TX, USA) was used according to manufacturer's instructions to isolate mRNA from cells growing in monolayer cell culture and a NanoDrop 2000c (Thermo Scientific, Wilmington, DE, USA) was used to quantify total RNA. A total of 0.5 µg of RNA was reversed transcribed into cDNA using iScript Reverse Transcription Supermix (Bio-Rad Hercules, CA, USA) according the manufacturer's instructions. RT PCR was then performed by Bio-Rad 96 well thermo cycler using QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA). Table 1 lists primers used for gene expression analysis.

Ribosomal RNA 18S was used for normalization. The cycle threshold (Ct) values for specific target gene and 18S of each cDNA test sample (test) and control (ctl) were measured and obtained using Opticon Monitor Analysis Software: Version 2.02 (Bio-Rad). In accordance with the delta delta Ct ($\Delta\Delta$Ct) method, relative transcript levels were calculated as $x = 2^{(-\Delta\Delta \text{ delta Ct})}$, where $\Delta\Delta Ct = \Delta E - \Delta C$, $\Delta E = Ct_{test} - Ct_{18S}$; $\Delta C = Ct_{ctl} - Ct_{18S}$.

Alcian blue staining and quantification

On Day 6 and 12, media was removed from each of the stable transfected ATDC5 clones and gently washed with sterile 1x PBS to remove detached/dead cells and excess media. ATDC5 cells were fixed in cold 100% methanol for 2 minutes at -20^{o}C and gently washed with 1x PBS. Samples were stained overnight with 500ul of 0.1% Alcian blue in 0.1M HCL pH = 1.0, plates were washed 3 times in milli-Q water (Millipore). Photos were taken with Nikon digital against a light box. Blue stains were extracted for quantification with 300 ul of 6.0 M GuHCl which was incubated overnight. Extracted color was measured in a 96 well plate at 620 nm using a spectrophotometer.

Cell number and viability assay

Transfected and untransfected ATDC5 cell lines were individually seeded at a density of 9000 cells/well in a 12 well culture plate. Over an 8 day time course, viable cell number was quantified for each cell line using Trypan blue and a hemocytometer. Cells were trypsinized and collected for counting on culture day 1, 2, 4 and 8. Parental ATDC5 cells treated with 10 ng/ml of TGF-β was used

as a positive control to account for increasing cell numbers characteristic to chondrogenesis.

Histology and safranin-O staining of primary PSFB cell pellets

Primary PSFB cell pellets were fixed using 4% paraformaldehyde in PBS (pH 7.4) for 24 hours at 4°C followed by dehydration in ethanol. Specimens were then cleared using xylene and embedded in paraffin. Individual sections (5 µm) were then cut and subsequently stained with safranin-O/fast green (for visualization of GAG) and counterstained with hematoxylin [1].

GAG quantification with dimethyl-methylene blue assay

After 3, 11 and 18 days in culture, primary PSFB cell pellets were digested in buffer containing papain (125 mg/mL), 100 mM phosphate, 10 mM EDTA and 10 mM cysteine. Using the digested lysates, a dimethylmethylene blue assay [1] was conducted to measure GAG content with a spectrophotometer (PerkinElmer, Norwalk, CT, USA). Bovine chondrotin sulfate was used to generate the standard curve utilized for GAG quantification. DNA content, as measured by Hoechst 33258 dye and a spectrofluorometer (QM-1; Photon Technology International, South Brunswick, NJ, USA), was used to control for cell number. Type I calf thymus DNA Sigma-Aldrich (St. Louis, MO, USA) was used to generate the standard curve utilized for DNA quantification.

Statistical analysis

All quantitative data are expresses as mean ±SDM (one standard deviation of the mean). Statistical analysis was conducted using a one-way ANOVA followed by a post-hoc test. Differences between groups were considered statistically significant when *p*-values were smaller than or equal to 0.05.

Results

Characterization of stably transfected ATDC5 clones

To understand the function of ECM protein MATN3 during chondrogenesis, we transfected the ATDC5 mouse chondroprogenitor cell line [1] to stably express WT-MATN3 or constructs carrying the MATN3 mutations associated with MED, SEMD, or HOA (Figure 1). Selective reagent G418 was used to isolate neomycin resistant WT and mutant MATN3 clones. With the exception of SEMD, all other selected WT and mutant MATN3 clones (WT3, M5, H2) exhibited relatively similar up regulation of MATN3 mRNA levels. These selected MATN3 expressing clones were thus subcultured and used for further analysis. Since the SEMD-MATN3 mutation is autosomal recessive and thus two copies of the allele must be present to cause a phenotype, we chose SEMD clone S5 (Figure 2A) which exhibited approximately 2 fold higher mRNA expression of the transgene relative to cells transfected with the dominant mutations. Western blotting and immunocytochemistry with anti V5 were used to further characterize the oligomerization and secretion behavior of each recombinant WT and mutant MATN3 protein expressed in ATDC5 cells. A 50 kDa monomer MATN3 band was observed in the lysate samples of WT-MATN3

transfected ATDC5 cells, while MATN3 protein secreted into the cell media formed primarily tetramers and trimers (Figure 2B) consistent with previous findings in primary bovine chondrocytes [14]. Additionally we detected dimer and monomer forms of MATN3 in transfected ATDC5 cell supernatants under non reducing conditions. Consistent with mRNA expression, SEMD-MATN3 protein was also produced at higher levels than WT and other mutant MATN3 proteins. However, a large amount of SEMD-MATN3 protein was detected in cell lysates rather than in cell supernatants. Oligomerization of MATN3 into tetramers was compromised by the MED mutation, since only trimers and dimers were detectable in cell supernatants (Figure 2B). Furthermore, immunocytochemistry showed retention of the recombinant SEMD protein within cell cytoplasm and intracellularly retained MATN3 protein localized within the nucleus was observed in ATDC5 cells expressing MED-MATN3 (Figure 2C). Comparably, such strong cellular retention of MATN3 protein was not apparent in HOA-MATN3 transfected ATDC5 cells (Figure 2C). Immunoblotting of recombinant MATN3 protein from these cell supernatants were comparable to that of supernatants from cells expressing WT-MATN3 (Figure 2B). We also demonstrate that ATDC5 cells expressing WT, MED and HOA-MATN3 proteins form cell aggregates and display a matrix, which closely resembles the chondrogenic phenotype of untransfected ATDC5 cells stimulated with TGF-β1 in culture for 6 days (Figure 3). Phase contrast microscopy showed ATDC5 cells transfected with SEMD exhibited unique morphology unlike any other MATN3 mutant clone. The presence of the SEMD mutant MATN3 mutation appears to induce ATDC5 cells

to form tight round clusters and aggregated together. Unlike the other transfected ATDC5 clones, the cellular phenotype of SEMD-MATN3 transfected cells appears to vary the most from untransfected parental ATDC5 cells that underwent chondrogenic cellular condensation in response to TGF-β1 treatment (Figure 3A). Despite their morphological differences, these transfected cell lines yielded a similar number of viable cells in a time course spanning 8 days (Figure 3B). All MATN3 transfected ATDC5 lines exhibited cell counts comparable to that of ATDC5 cells treated with TGF-β1, which were undergoing chondrogenic cellular condensation.

ATDC5 cells stably transfected with MATN3 undergoes spontaneous chondrogenesis

To further characterize the chondrogenic properties of MATN3, we analyzed mRNA levels of chondrogenic markers type II collagen (Col2a1) and aggrecan in ATDC5 cells stably transfected with WT and mutant MATN3 genes. Our initial time point for this analysis was 24 hours (day 0) after cells were plated. Stably transfected ATDC5 cells expressing WT-MATN3 significantly up regulated Col2a1 mRNA levels by more than 2 fold compared to untransfected ATDC5 cells (Figure 4A). Similarly, WT-MATN3 stimulated aggrecan mRNA expression by 6 fold on day 0 (Figure 4B). In contrast, ATDC5 cells carrying MED, SEMD and HOA point mutations not only suppressed elevation of Col2a1 mRNA on day 0, but they also significantly down regulated Col2a1 mRNA levels compared to untransfected ATDC5 cells (Figure 4A). Additionally, up regulation of aggrecan

levels was also suppressed in ATDC5 cells transfected with MED and SEMD MATN3 mutations on day 0. Interestingly, cells carrying the HOA-MATN3 mutation exhibited a trend of decreasing aggrecan expression relative to WT-MATN3 transfected ATDC5 cells (Figure 4B).

MED and SEMD mutations severely alter MATN3 induced chondrogenesis

Alcian blue staining was used to measure proteoglycan content produced by ATDC5 cells expressing WT or mutant MATN3 genes. On day 6 and day 12, untransfected ATDC5 cell cultures did not stain positive for Alcian blue (Figure 5A, 5E) indicating that these cells did not undergo chondrogenesis spontaneously. However substantial blue staining was observed in cells stably transfected with WT-MATN3, indicating substantial proteogylcan accumulation. Furthermore, while ATDC5 cells expressing the MED-MATN3 mutation resulted in a slight reduction in staining relative to cells expressing WT-MATN3, only the SEMD-MATN3 expressing cells showed significant decrease in alcian blue staining on both day 6 and 12 (Figure 5A, 5E). The HOA-MATN3 transfected cells did not exhibit a substantial weakening of alcium blue staining patterns relative to WT-MATN3 expressing cells on these days. However, histological analysis of primary PSFBs expressing the SEMD-MATN3 mutation, and those expressing the HOA-MATN3 mutation, showed significantly lower safranin-O staining intensities, relative to WT-MATN3 transfected cells, on all 3 days in the peripheral regions of the cell pellet suggesting an overall attenuation of chondrogenesis (Figure 6). Similarly, PSFBs expressing the same MATN3

mutations yielded significantly lower GAG quantities than that observed in primary PSFBs transfected with the WT-MATN3 gene construct (Figure 7) once again suggesting that these MATN3 mutations may greatly hinder proteogylcan accumulation in cells undergoing chondrogenesis.

Gene expression analysis using real-time PCR demonstrated that WT-MATN3 in ATDC5 cells stimulated an increase in aggrecan mRNA from 7 fold (day 6) to more than 13 fold (day 12) compared to untransfected ATDC5 cells (Figure 5B, 5F). Furthermore, WT-MATN3 expression by ATDC5 cells resulted in a transient up regulation in Col2a1 mRNA peaking at day 6 and returning to basal level by day 12 (Figure 4C, 4G). We also observed that WT-MATN3 did not strongly alter Col10a1 expression in ATDC5 cells at day 6 but by day 12. At day 6 and day 12, both MED and SEMD mutations dramatically suppressed MATN3's ability to stimulate aggrecan expression (Figure 5B, 5F). The SEMD mutation seemed to reduce even basal expression of aggrecan. Additionally, the MED mutation suppressed MATN3's stimulation of Col2a1 at day 6 and day 12. The SEMD mutation exhibited severe down regulated Col2a1 mRNA levels that were almost undetectable by real-time PCR on both days. Although the HOA mutation delayed up regulation of Col2a1 on day 6, it stimulated Col2a1 expression later on day 12 and aggrecan expression on both day 6 and day 12 (Figure 5B, 5F, 5G). Additionally, the HOA mutation did not alter the inhibitory effect of MATN3 on Col10a1 mRNA expression (Figure 5D, 5H). Interestingly, while MED and SEMD mutations negatively affected the expression of Col2a1 and aggrecan, which are indicators for chondrogenesis, the hypertrophic marker

Col10a1 was up regulated significantly in ATDC5 cells expressing the MED and SEMD-MATN3 point mutations by day 6, and remained elevated at day 12.

In addition to analyzing the expression of hypertrophic marker Col10a1, Ihh gene expression was also analyzed in ATDC5 cells stably transfected with WT and mutant MATN3 constructs. SEMD and HOA mutations of MATN3 increased Ihh mRNA levels at day 6 (Figure 8A). However, HOA-MATN3 expressing ATDC5 cells lost its abnormally high Ihh expression by day 16 while MED-MATN3 and SEMD-MATN3 expressing cells retained high Ihh mRNA levels (Figure 8B).

ATDC5 cells transfected with HOA-MATN3 exhibit disregulated MMP-9, MMP-13 and ADAMTS-5

To better understand how HOA-MATN3 increases risk of arthropathy, we wanted to explore the possibility that this mutation can disregulate the expression of major chondrocyte markers, other than anabolic markers, that are also important for homeostasis. Interestingly, we discovered that ATDC5 chondroprogenitors transfected with WT-MATN3 exhibited elevated expression of cartilage resorption marker MMP-9 (Figure 9A). In contrast, HOA-MATN3 was incapable of stimulating this marker. However, both WT-MATN3 transfected cells and HOA-MATN3 transfected cells showed elevated mRNA levels of a second resorption marker that is endogenously expressed in cartilage: MMP-13 (Figure 9B). Furthermore, ADAMTS-5, a third cartilage resorption marker and aggressive

aggrecanase was highly elevated in HOA-MATN3 transfected cells relative to both untransfected and WT-MATN3 transfected cells (Figure 9C).

WT-MATN3, but not HOA-MATN3, enhances IL-1Ra expression

In a previous study we demonstrated that MATN3 depends on the anti-inflammatory cytokine IL-1Ra to regulate various cartilage homeostasis markers [26]. To determine if MATN3's induction of IL-1Ra affects the process of chondroprogenitor cell differentiation, we first used real-time PCR to verify that MATN3 is indeed capable of stimulating IL-1Ra expression in ATDC5 chondroprogenitor cells. Gene expression analysis via real-time PCR revealed that WT-MATN3, but not HOA-MATN3, stimulated a 17 fold increase in IL-1Ra expression compared to untransfected ATDC5 control cells. HOA-MATN3 only induced a 3 fold induction of IL-1Ra relative to parental untransfected ATDC5 cells indicating that the HOA associated mutation hinders MATN3's ability to stimulate IL-1Ra (Figure 10A).

Knocking down IL-1Ra diminishes MATN3's induction of chondrogenesis markers in ATDC5 cells

To see if MATN3's induction of chondrogenic marker expression in ATDC5 murine chondroprogenitors is dependent on its stimulation of IL-1Ra, we used a small interfering RNA (siRNA) that targets and transiently suppresses all transcriptional variants of murine IL-1Ra to specifically knock down this gene in WT-MATN3, HOA-MATN3 and parental ATDC5 cell lines. A non-silencing

scrambled siRNA was used as a negative control. Gene expression analysis 72 hours after transfection shows that the IL-1Ra siRNA diminished IL-1Ra mRNA levels in all three transiently transfected cell lines (Figure 10B, right panel) while the non-silencing control did not (Figure 10B, left panel). All IL-1Ra siRNA transfected cells exhibit no difference in IL-1Ra mRNA levels as a result of this knock down, effectively eliminating MATN3's stimulation of this molecule.

Using these IL-1Ra silenced cells as biological models, we compared chondrogenesis markers Acan, Col2a1 and Sox9 mRNA levels between parental ATDC5 cells and cells over-expressing the WT or HOA-MATN3 gene. WT-MATN3 transfected ATDC5 cells exhibited enhanced Acan mRNA levels in comparison to parental ATDC5 cells (Figure 11A, left panel). In contrast, cells expressing HOA-MATN3 reduced the expression of Acan relative to parental ATDC5 cells. Knocking down IL-1Ra significantly reduced the basal expression of Acan in all cell lines including WT-MATN3 transfected cells (Figure 11A, right panel). Similar to Acan, WT-MATN3 also enhanced Col2a1 mRNA expression by approximately 4.8 fold in ATDC5 cells in comparison to untransfected parental ATDC5 control cells while HOA-MATN3 further reduced mRNA levels of this chondrogenesis marker by more than 4 fold in ATDC5 cells (Figure 11B, left panel). As with Acan, knocking down IL-1Ra caused a significant reduction in the Col2a1 mRNA levels observed in WT-MATN3 transfected cells (Figure 11B, right panel). Finally, we quantified the mRNA levels of chondrogenic transcription factor Sox9 in these cell lines. WT-MATN3 transfected ATDC5 cells exhibited a 1.6 fold upregulation in Sox9 mRNA levels relative to untransfected parental

controls. We did not observe significant alterations in Sox9 mRNA levels in HOA-MATN3 transfected cells relative to untransfected controls (Figure 11C, left panel). Knocking down IL-1Ra caused a significant reduction in Sox9 expression in WT-MATN3 transfected cells while HOA-MATN3 transfected cells remained largely unchanged (Figure 11C, right panel). As with Acan and Col2a1, WT-MATN3 stimulation of Sox9 is IL-1Ra dependent.

MATN3 stimulates chondrogenesis markers in primary human CPCs

Next we inquired whether our observations in murine cells were consistent in human cells. To do this, we utilized several patient cartilage samples to test if MATN3 can enhance chondrogenesis marker expression in primary human CPCs isolated from the cartilage tissue of a 57 year old female patient and a 76 year old female patient. Patient samples were not pooled. Using mRNA analysis, we first verified the expression of known stem cell markers in individually isolated human CPC colonies in a patient specific manner. As expected, CPC colonies isolated by means of differential adhesion to fibronectin exhibited elevated expression of the fibronectin receptor CD49e (between 3 to 5 fold) relative to PHC controls (Figure 12A). Likewise, expression of MSC marker CD44 was elevated 3 to 5 fold in CPC colonies compared to controls (Figure 12A). MSC marker CD166 also showed a 6 to 7 fold elevation in expression in tested CPC colonies (Figure 12A). To further verify that these progenitor-like cells are different from mature articular chondrocytes, we also quantified the basal mRNA expression of chondrocyte markers Col2a1 and Sox9 (Figure 12B) between CPC

colonies and PHC controls in a patient specific manner and found that they were generally expressed at significantly higher levels in PHCs, as expected. Primary human CPCs treated with recombinant human MATN3 protein (rhMATN3) exhibited elevated chondrogenesis marker expression including ACAN, COL2A1 and SOX9 relative to untreated cells (Figure 13).

Discussion

Extracellular matrix proteins may provide a microenvironment that is much needed to facilitate chondrogenic differentiation during cartilage development and/or healing. MATN3 is a protective cartilage specific matrix protein whose physiological role remains to be fully elucidated [25]. Mutations in the MATN3 gene are associated with abnormalities in cartilage development (i.e. MED and SEMD) and HOA. Previously, it was shown that MATN3 can have a synergistic effect on TGF-β induced chondrogenesis of synovial fibroblasts [1]. In the present study, there were several goals. The first goal was to determine whether wild-type MATN3 or three MATN3 mutations closely associated with MED, SEMD or HOA can induce chondrogenesis in chondroprogenitor cells, which are MSCs of the chondrogenic lineage that have recently become of great interest to regenerative medicine due to their ability to differentiate into mature articular chondrocytes. The second goal of this study was to identify potential disease causing effects of the HOA-MATN3 on chondroprogenitors that can help explain why patients with this mutant gene are at a greater risk of developing arthropathy. The final goal was to understand how MATN3's stimulation of the

anti-inflammatory cytokine IL-1Ra influences its regulatory capacity in chondroprogenitors.

To accomplish these goals, four cell lines were generated by stably transfecting ATDC5 murine chondroprogenitor cells with cDNA constructs carrying the wild-type or mutant MATN3 genes. All generated cell lines expressed WT or mutant MATN3 gene at a similar level except SEMD since this particular mutation is autosomal recessive in nature and thus may require elevated expression levels to have a phenotypic effect. MATN3 monomer protein levels from cell lysates of each clone confirmed that WT, MED and HOA clones were producing MATN3 protein. Consistent with a previous study, we found that the SEMD-MATN3 mutation caused much intracellular retention of the mutant protein [14] which may account for why this mutation in particular causes severe chondrodysplasia in people. Immunocytochemical analysis confirmed that HOA was the only MATN3 mutation that did not cause some degree of abnormal MATN3 protein secretion and oligomerization relative to WT-MATN3 expressing cells.

While only TGF-β1 stimulated untransfected ATDC5 cells condensed into the chondrogenic phenotype, we observed that all MATN3 transfected lines underwent varying degrees of spontaneous chondrogenic cellular condensation without TGF-β1 stimulation suggesting that MATN3 expression alone is sufficient to promote chondrogenesis in chondroprogenitors. Quantifying proteoglycan content via alcian blue staining showed that all four transfected ATDC5 clones stained positively with alcian blue, with WT-MATN3 and HOA clones exhibiting

the strongest staining pattern, whereas the lowest positive staining was observed in the case of SEMD; and untransfected ATDC5 cells remained unstained altogether. Interestingly, the discrepancy in alcian blue staining intensities across the transfected ATDC5 clones seems to be reflective of the severity of each MATN3 mutation. For example, clinically the SEMD mutation is phenotypically the most severe cartilage abnormality of the three mutations with MED coming in second and HOA being third. Furthermore, HOA is classified as an arthropathy in which the effects are relatively mild in comparison to MED and SEMD, which are two severe forms of chondrodysplasia. We believe this may be the reason why HOA-MATN3 expressing cells show an alcian blue staining intensity that is quite similar to that of WT-MATN3 expressing cells while the much more severe SEMD mutation seems to noticeably attenuate proteoglycan content.

Gene expression analysis via real-time PCR revealed that all mutant MATN3 transfected ATDC5 lines exhibited lower aggrecan and type II collagen expression relative to WT-MATN3 transfected cells 24 hours after culture. It was interesting to find that the HOA-MATN3 line seemed to recover from this state of suppressed aggrecan and type II collagen expression by culture day 6 and 12, respectively. This strongly suggests that the HOA associated MATN3 mutation delays the expression of chondogenesis markers in progenitor cells. Perhaps this phenomenon can also help explain why primary PSFBs stably transfected with HOA-MATN3 exhibited weak safranin-O staining in the pericellular region compared to WT-MATN3 transfected cells. In contrast to HOA-MATN3, the

chonrodysplasia associated mutations exhibited suppression of type II collagen and aggrecan for all tested time points, with the exception of MED-MATN3 showing a mild upregulation of type II collagen by culture day 12. These severe chondrodysplasia mutations appear to ultimately hinder MATN3's stimulation of anabolic markers characteristic of normal chondogenic differentiation and consequently attenuates alcian blue staining, which corresponds to proteoglycan content. Furthermore, the significant elevation of hypertrophic marker Col10a1 in only the MED and SEMD-MATN3 transfected cells suggest that these mutations may also disrupt the process of normal chondrogenesis of progenitor cells by promoting premature hypertrophy. Conversely, WT-MATN3 transfected cells exhibited an overall decreasing trend of Col10a1 expression by culture day 12 relative to untransfected ATDC5 cells suggesting that WT-MATN3 may endogenously help delay chondrocyte hypertrophy.

Upon quantifying expression of Ihh, a recently reported marker of chondrocyte hypertrophy [30], we saw a similar trend as that observed with Col10a1. SEMD transfected ATDC5 cells exhibited elevated Ihh mRNA levels relative to untransfected ATDC5 cells at day 6 and MED transfected cells showed a similar trend at day 16. It is worth noting that at day 6, the HOA-MATN3 expressing line also showed significant upregulation of Ihh; however this effect subsided by day 16. Overall it is clear that, of the four transfected ATDC5 lines, only the WT-MATN3 transfected line consistently exhibits low expression of hypertrophic markers Col10a1 and Ihh at all observed time points while these markers are elevated in cell lines expressing MATN3 mutations. These findings

suggest that premature chondrocyte hypertrophy may not only be a distinct characteristic but also a potential disease causative factor in patients carrying the MED and SEMD causing MATN3 mutations.

To better understand the HOA associated MATN3 mutation and its potential disease causing effects, we measured its ability to regulate several cartilage resorption markers in differentiating chondroprogenitors and found that this mutation significantly reduced the expression of MMP-13; however, it also increased the expression of the aggressive aggrecanase ADAMTS-5, relative to WT-MATN3 expressing ATDC5 cells. Since the HOA mutation also appears to severely hinder MATN3's ability to stimulate IL-1Ra, its induction of ADAMTS-5 is not surprising because we have shown in a previous study that knocking down IL-1Ra can elevate ADAMTS-5 expression *in vitro* [26]. Furthermore, we also observed the importance of IL-1Ra in mediating the chondrogenic effects of WT-MATN3 on ATDC5 chondroprogenitors, as knocking down IL-1Ra inhibited WT-MATN3's induction of major chondrogenesis markers aggrecan, type II collagen and Sox9. These findings suggest that WT-MATN3's stimulation of IL-1Ra may play a pivotal role during chondrogenesis by promoting chondrocyte marker expression in differentiating chondroprogenitors, a phenomenon that is severely attenuated by HOA-MATN3 (Figure 14). Interestingly, studies have previously reported that IL-1 pathway activation inhibits chondrogenesis processes in MSCs [31, 32]. Perhaps attenuation of the IL-1 pathway by IL-1Ra is the mechanism by which WT-MATN3 promotes chondrogenesis. This may also help explain why progenitors containing HOA-MATN3, which exhibited much weaker expression of

IL-1Ra, underwent delayed chondrogenesis relative to WT-MATN3 expressing cells.

Finally, our studies using primary human chondroprogenitors isolated through differential adhesion to fibronectin illustrates that MATN3's stimulation of chondrogenesis markers in chondroprogenitors is not only observed in the murine model, but also observed in human cells. The translatability of this pathway to human cells emphasizes the overall importance of MATN3 to the process of cartilage development and healing in our bodies. Moreover, MATN3's ability to stimulate chondrogenesis of chondroprogenitor cells may explain why mice functionally deficient in this gene experience early onset of OA [25]. MATN3's stimulation of progenitor cell differentiation may be a natural phenomenon that helps replace damaged chondrocytes and may overall promote tissue healing proving it to be useful for future endeavors in cartilage regenerative medicine.

Conclusion

This study presents evidence supporting the hypothesis that cartilage ECM protein MATN3 may provide a microenvironment suitable for promoting chondrogenic differentiation. We have demonstrated that while ATDC5 cells that constituently express MATN3 (WT or mutant) can undergo varying degrees of spontaneous chondrogenesis, cells transfected with pcDNA constructs containing the severe chondrodysplasia causing MED or SEMD-MATN3 mutations show significant disregulation of chondrogenesis markers. These two

MATN3 mutations also exhibit enhanced markers of terminal differentiation and hypertrophy. We also demonstrate that the HOA associated mutation disregulated MATN3's ability to stimulate IL-1Ra as well as several downstream chondrogenesis markers. We propose that HOA-MATN3's inability to adequately stimulate IL-1Ra may be responsible for the delayed process of chondrogenesis observed in progenitor cells expressing this mutation.

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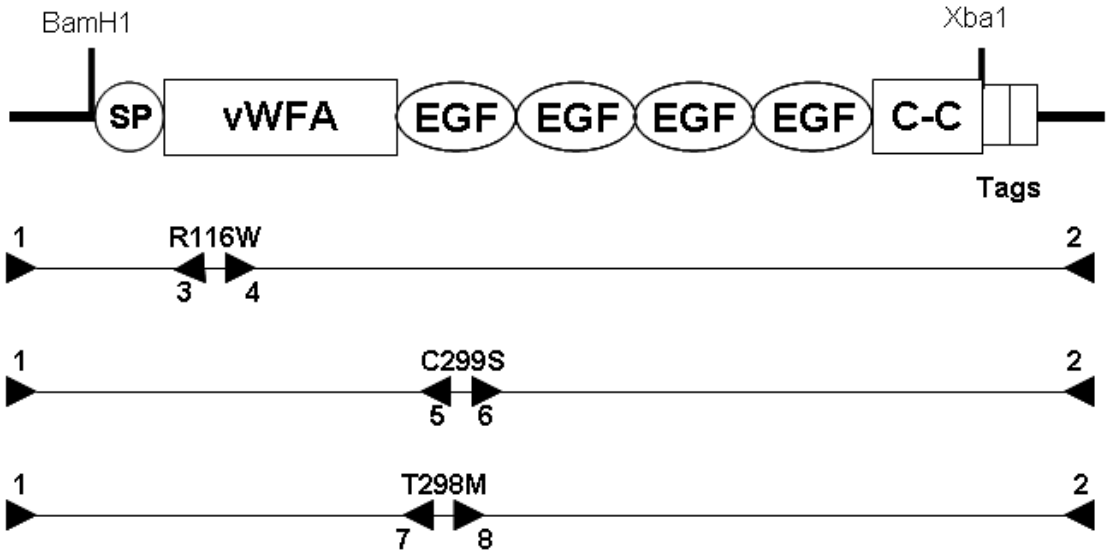
TABLE 1

Species	Gene	Forward sequence	Reverse sequence
Human	CD44	5'-CCCCAGCAACCCTACTGATG-3'	5'-TTGCCTCTTGGTTGCTGTCT-3'
Human	CD49e	5'-GGCTTCAACTTAGACGCGGA-3'	5'-ATTCAATGGGGGTGCACTGT-3'
Human	CD166	5'-AAGGTTTTCCAAAGCCAGCC-3'	5'-TCACCTTTTCTCTGTTTTCATTAG-3'
Human	ACAN	5'-ACCAGACGGGCCTCCCAGAC-3'	5'-TGGCTCTGCCCCAGAGGGAC-3'
Human	COL21A	5'-TGAGGGCGCGGTAGAGACCC-3'	5'-TGCACACAGCTGCCAGCCTC-3'
Human	SOX9	5'-GGACCAGTACCCGCACTTGCA-3'	5'-GTTCTTCACCGACTTCCTCCGCCG-3'
Murine	Acan	5'-CAGTGCGATGCAGGCTGGCT-3'	5'-CCTCCGGCACTCGTTGGCTG-3'
Murine	ADAMTS-5	5'-AGCAAGAATGCGGCCACGAC-3'	5'-AACGTCTGCCATTCCCAGGGT-3'
Murine	Col2a1	5'-CACACTGGTAAGTGGGGCAAGACCG-3'	5'-GGATTGTGTTGTTTCAGGGTTCGGG-3'
Murine	Col10a1	5'-GCCAGGAAAGCTGCCCCACG-3'	5'-GAGGTCCGGTTGGGCCTGGT-3'
Murine	IL-1Ra	5'-ACCCATGGCTTCAGAGGCAGC-3'	5'-GCCCCCGTGGATGCCCAAG-3'
Murine	Ihh	5'-GCGCAGCTCTGAGCGCTTCA-3'	5'-GCCTTCGGTCACCCGCAGTT-3'
Murine	MMP-9	5'-CTGTTCAGCAAGGGGCGTGT-3'	5'-CAAGGGCACTGCAGGAGGTC-3'
Murine	MMP-13	5'-GGACCTTCTGGTCTTCTGGC-3'	5'-GGATGCTTAGGGTTGGGGTC-3'
Murine	Sox9	5'-AACTTCTGTGGGAGCGACAA-3'	5'AAACAGAGAACGAAACCGGG-3'

Table 1. Forward and reverse primer sequences used for Real-Time PCR

FIGURE 1



Primers	Sequence 5' to 3'	Purpose
1	TAATACGACTCACTATAGGG	T7, amplifying inserts
2	GAGGAGAGGGTTAGGGATAGGCTTA	BGH, amplifying inserts
3	AAAGAACAACCTGGGTGGCAGTCATGA	Introducing MED mutation
4	TCATGACTGCCACCCAGGTGGTTCTTT	Introducing MED mutation
5	ATCAATGGCTGAACTCGTTTTCCCATCAGC	Introducing SEMD mutation
6	GGGAAAACGAGTTCAGCCATTGATAAGTGT	Introducing SEMD mutation
7	ATCAATGGCTGAACACATTTTCCCATCAGC	Introducing HOA mutation
8	GGGAAAATGTGTTCAGCCATTGATAAGTGT	Introducing HOA mutation

Figure 1: Introduction of MED, SEMD and HOA associated mutations into the MATN3 gene. The relative location of the primers used to produce pcDNA3.1/V5-6xHis carrying WT-MATN3 and various MATN3 point mutations are shown below the schematic model of MATN3. The primer sequences and their purpose are listed in the table. SP: signal peptide; C-C: coil-coil domain; Tags: V5 and 6xHis.

FIGURE 2

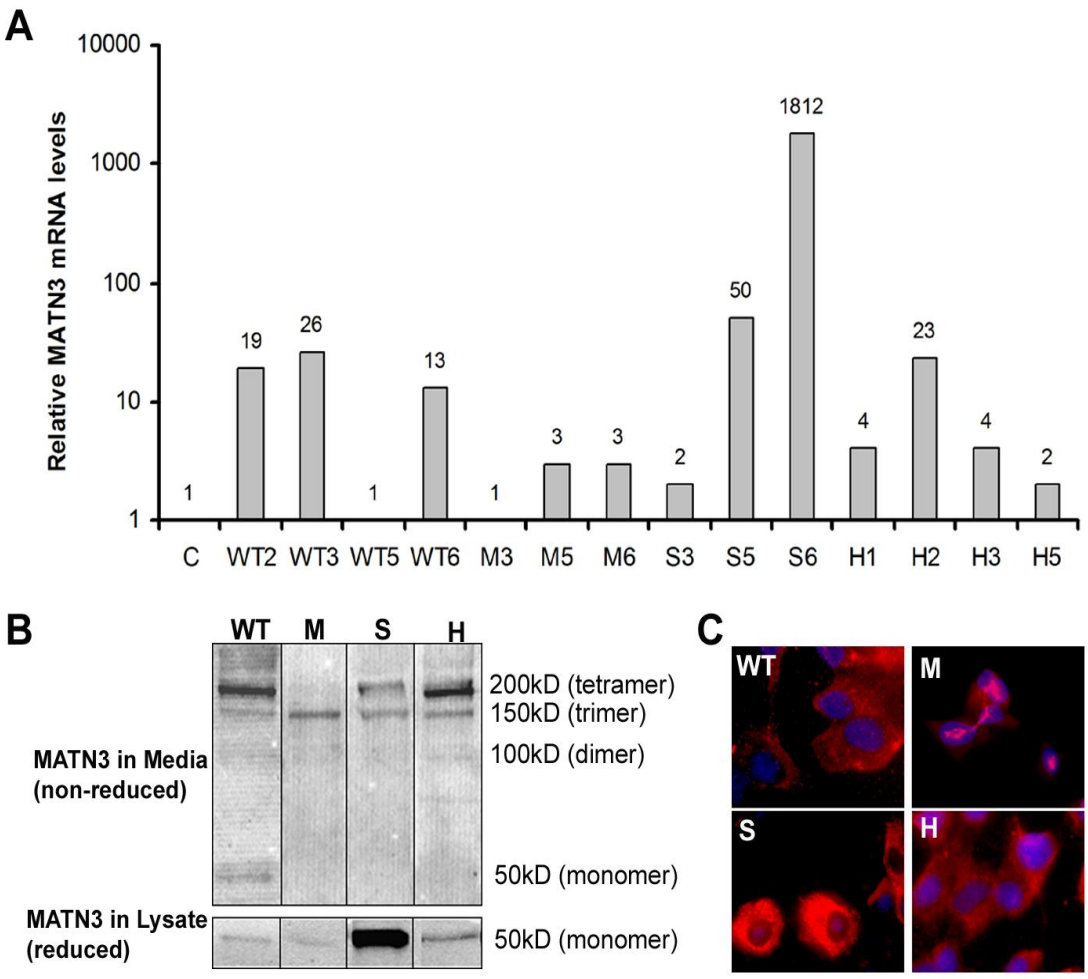

A
Relative MATN3 mRNA levels
10000
1000
100
10
1
1
19
26
1
13
1
3
3
2
50
1812
4
23
4
2
C
WT2
WT3
WT5
WT6
M3
M5
M6
S3
S5
S6
H1
H2
H3
H5
B
WT
M
S
H
MATN3 in Media
(non-reduced)
200kD (tetramer)
150kD (trimer)
100kD (dimer)
50kD (monomer)
MATN3 in Lysate
(reduced)
50kD (monomer)
C
WT
M
S
H

Figure 2: Characterization of MATN3 overexpression in stably transfected ATDC5 murine chondroprogenitor cells. Relative MATN3 mRNA expression of ATDC5 cells stably transfected with wild-type (WT) MATN3 and MATN3 mutations found in genetic disorders MED (M), SEMD (S) and hand osteoarthritis (H) (**A**). Western blot analysis using anti V5 to detect recombinant MATN3 protein in cell lysates under reducing conditions and cell media under non-reducing condition (**B**). Immunocytochemical analysis using V5 antibody was utilized to visualize the location of MATN3 recombinant protein as it is secreted or retained by stably transfected ATDC5 cell lines (**C**).

FIGURE 3

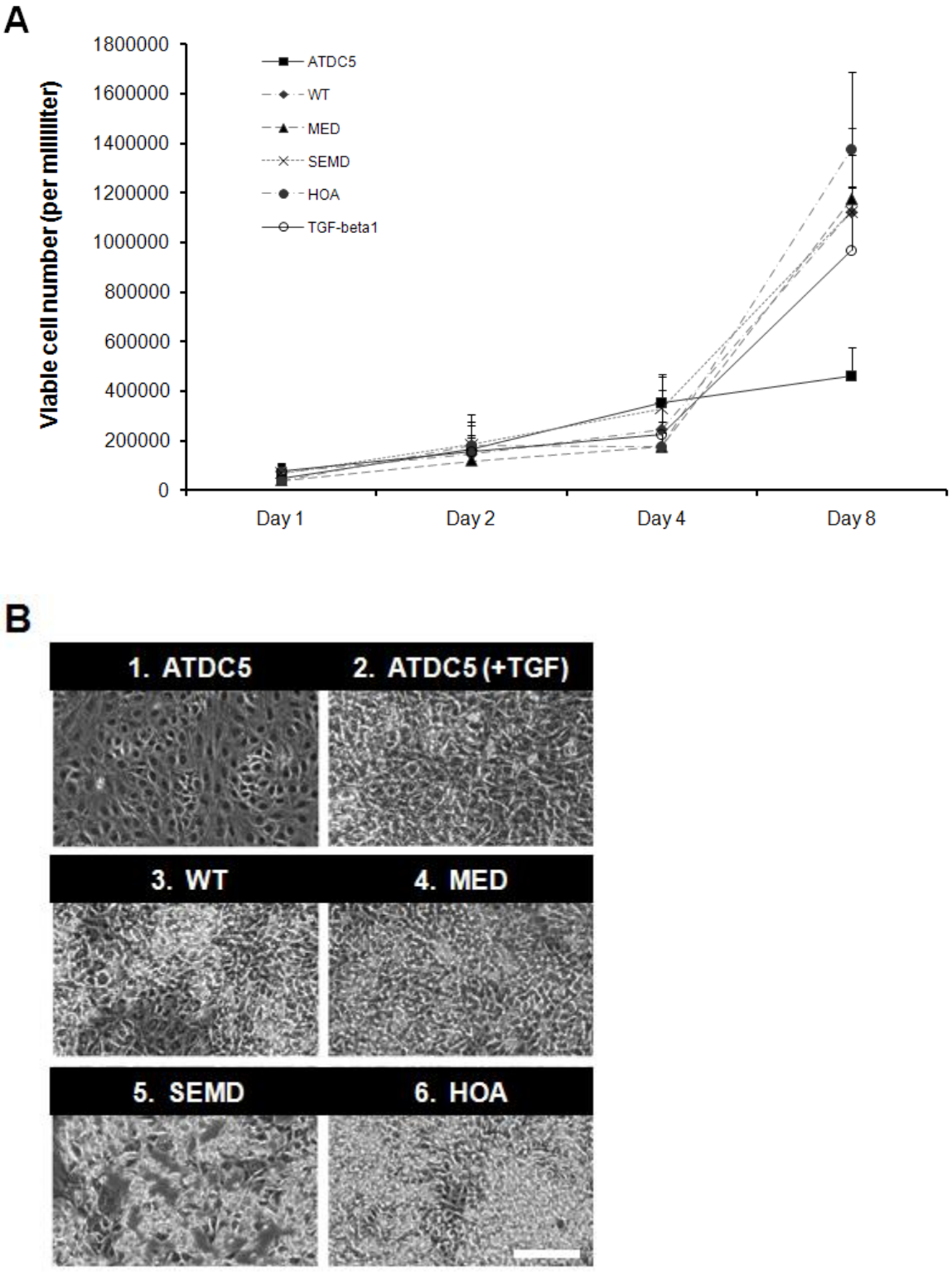

A
Viable cell number (per milliliter)
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
ATDC5
WT
MED
SEMD
HOA
TGF-beta1
Day 1
Day 2
Day 4
Day 8
B
1. ATDC5
2. ATDC5 (+TGF)
3. WT
4. MED
5. SEMD
6. HOA

Figure 3: Cell proliferation and morphology of chondroprogenitors expressing WT and mutant MATN3 genes. Number of viable cells as counted from the parental ATDC5 line and each transfected ATDC5 line, in a time span of 8 culture days **(A)**. “TGF-beta 1” data points refer to parental ATDC5 cells treated with 10 ng/ml of TGF-β1. Phase-contrast micrographs of day 6 untransfected ATDC5 cells without (1) or with 10 ng/ml TGF-β1 (2) and ATDC5 cells stably transfected with the WT-MATN3 construct (3) or MED (4), SEMD (5), and HOA (6) mutant MATN3 constructs **(B)**. Scale bar = 100mm.

FIGURE 4

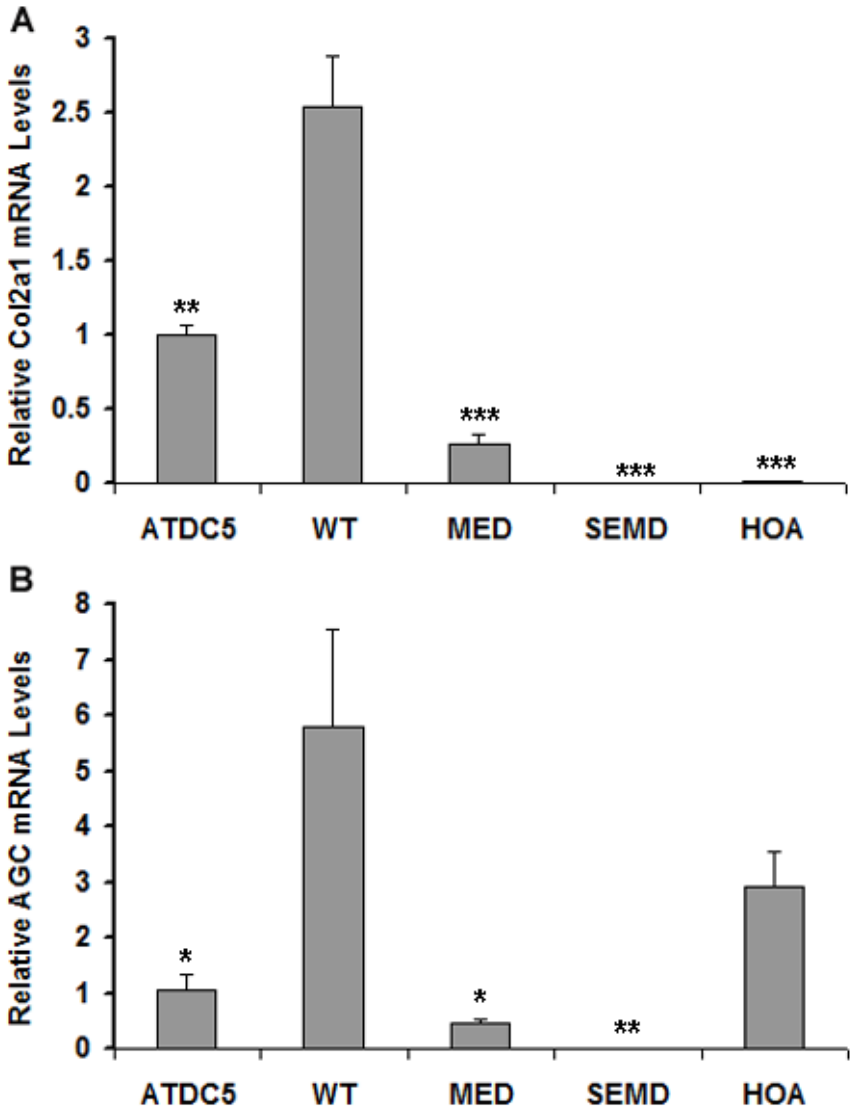

A
Relative Col2a1 mRNA Levels
3
2.5
2
1.5
1
0.5
0
**
***
***
***
ATDC5
WT
MED
SEMD
HOA
B
Relative AGC mRNA Levels
8
7
6
5
4
3
2
1
0
*
*
**
ATDC5
WT
MED
SEMD
HOA

Figure 4: MATN3 induction of chondrogenesis markers in chondroprogenitor cells. Relative mRNA expression levels of Col2a1 (**A**) and aggrecan (**B**) in WT and mutant MATN3 stably transfected and untransfected ATDC5 cells as analyzed by real-time PCR on day 0 (24 hours post plating). Experiments were done in biological triplicates. Single asterisks ($p \leq 0.05$), double asterisks ($p \leq 0.01$) and triple asterisks ($p \leq 0.005$) indicate statistically significant differences from WT MATN3 transfected ATDC5 cells.

FIGURE 5

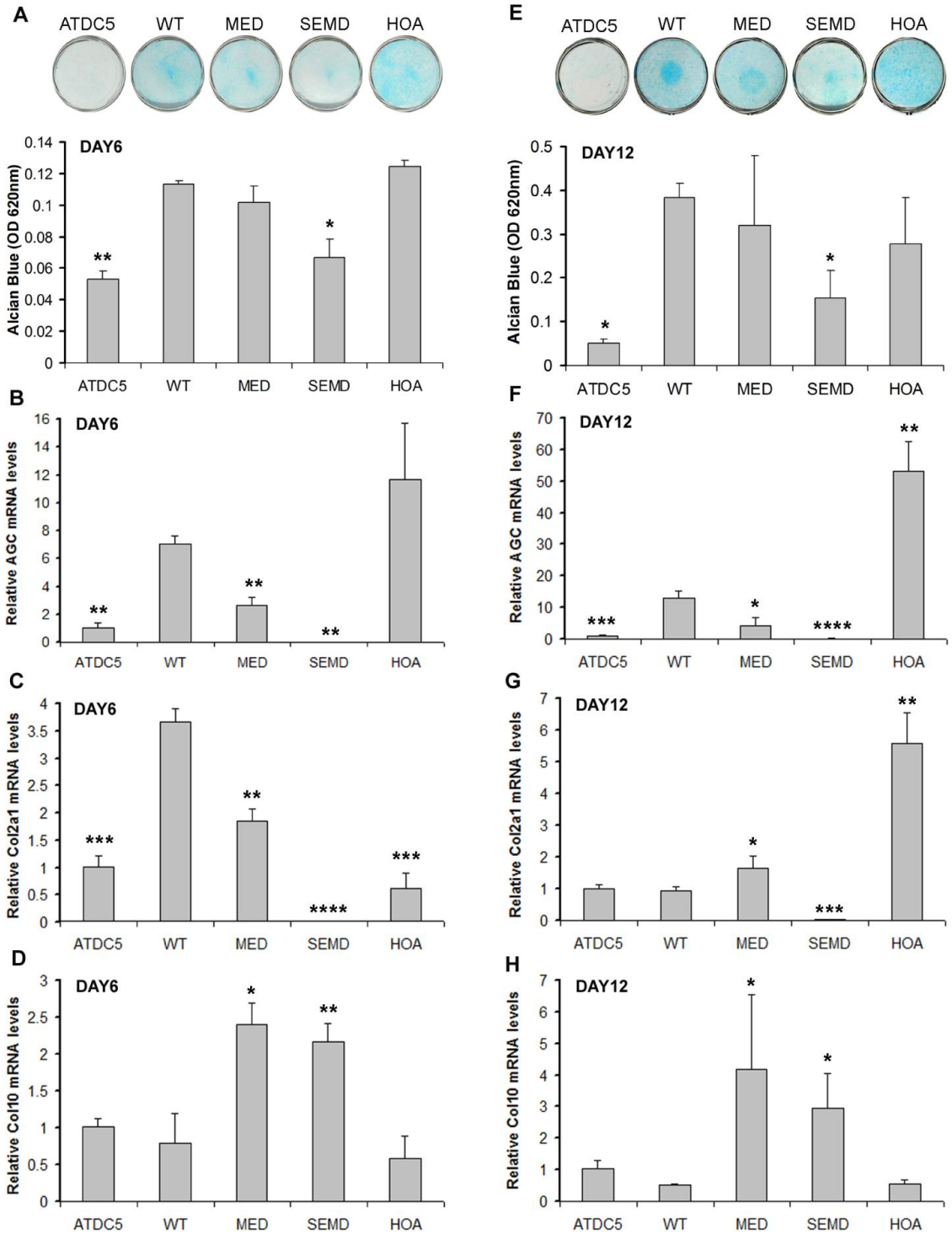

A
ATDC5
WT
MED
SEMD
HOA
DAY6
Alcian Blue (OD 620nm)
B
Relative AGC mRNA levels
C
Relative Col2a1 mRNA levels
D
Relative Col10 mRNA levels
E
DAY12
F
G
H

Figure 5: **The effect of MED, SEMD and HOA point mutations on MATN3 induced spontaneous chondrogenesis of stably transfected ATDC5 cells.** ATDC5 cells were fixed and stained for proteoglycan accumulation using alcian blue on day 6 (**A**) and day 12 (**E**). Optical density of alcian blue extracted with 6M GuHCl was quantified at 620nm. Relative mRNA expression of aggrecan (**B, F**), Col2a1 (**C, G**) and Col10a1 (**D, H**) was analyzed by real-time PCR in WT and mutant MATN3 transfected and untransfected ATDC5 cells on day 6 and 12. Experiments were done in biological triplicates. Single asterisks ($p \leq 0.05$), double asterisks ($p \leq 0.01$), triple asterisks ($p \leq 0.005$) and quadruple asterisks ($P \leq 0.0005$) indicate statistically significant differences from WT-MATN3 transfected ATDC5 cells.

FIGURE 6

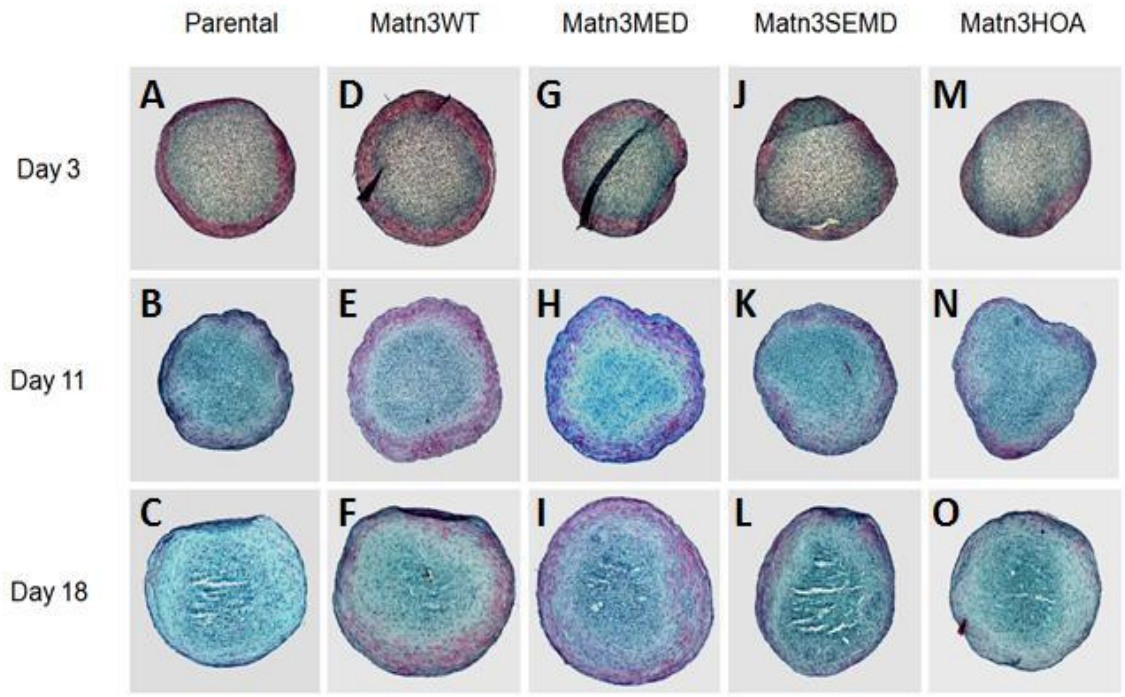

Parental
Matn3WT
Matn3MED
Matn3SEMD
Matn3HOA
Day 3
Day 11
Day 18
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 6. Safranin-O staining of primary porcine synovial fibroblasts transfected with WT or mutant MATN3 gene constructs. Extent of chondrogenesis of untransfected **(A, B, C)**, WT-MATN3 transfected **(D, E, F)**, MED MATN3 transfected **(G, H, I)**, SEMD MATN3 transfected **(J, K, L)** and HOA MATN3 transfected **(M, N, O)** porcine PSF pellets as indicated by Safranin-O staining. **Note: Panels A – F were previously published in **Osteoarthritis and Cartilage (2008) 16, 1110-1117**. These specific panels are shown here purely as a reference for the purpose of comparing the extent of chondrogenesis in WT-MATN3 transfected cells vs. MED, SEMD and HOA MATN3 transfected cells. Panels G – O are previously unpublished original images.*

FIGURE 7

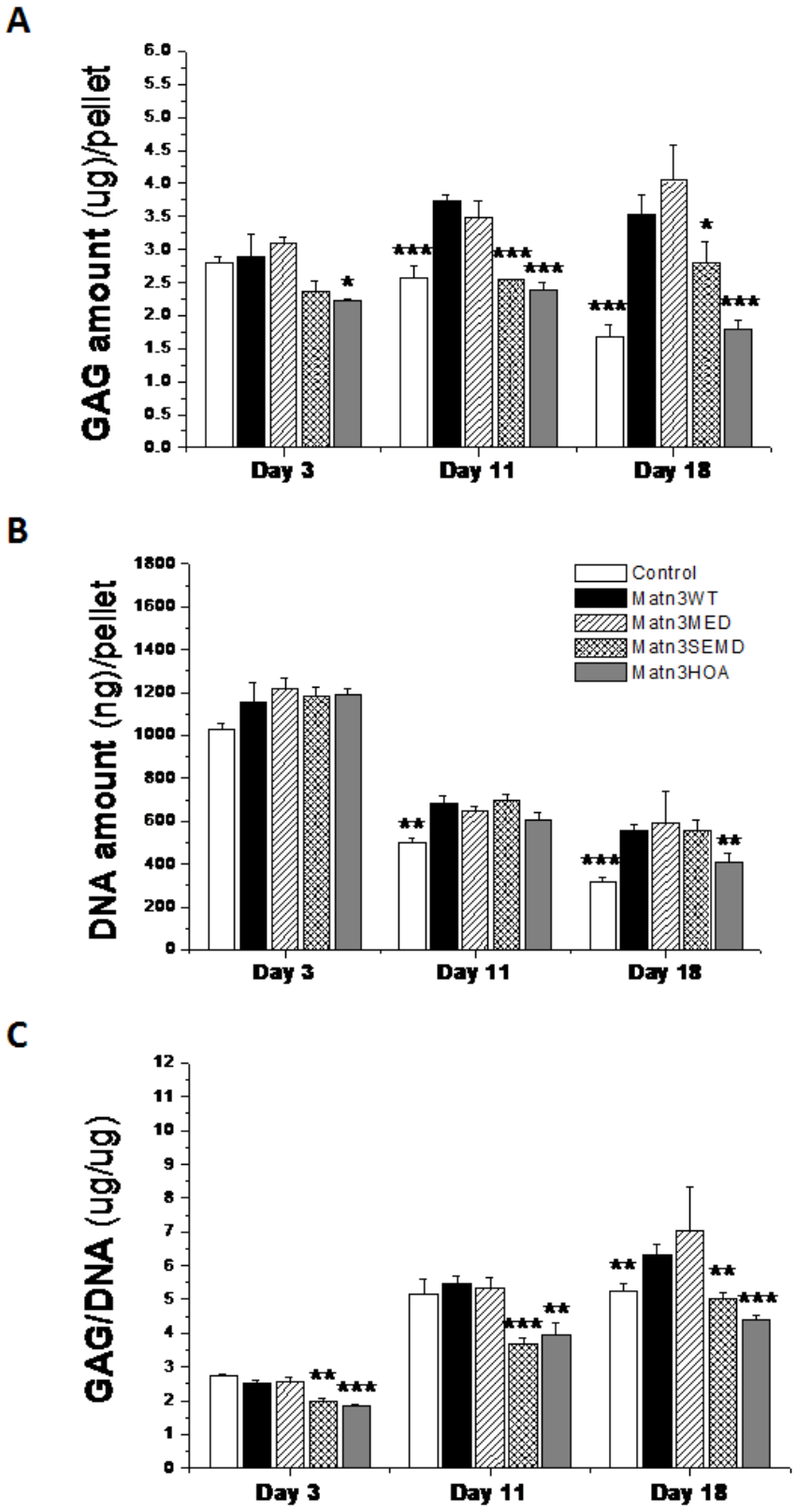

A
GAG amount (ug)/pellet
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Day 3
Day 11
Day 18
B
DNA amount (ng)/pellet
1800
1600
1400
1200
1000
800
600
400
200
0
Control
Matn3WT
Matn3MED
Matn3SEMD
Matn3HOA
Day 3
Day 11
Day 18
C
GAG/DNA (ug/ug)
12
11
10
9
8
7
6
5
4
3
2
1
0
Day 3
Day 11
Day 18

Figure 7. Quantification of glycosaminoglycan content in primary porcine synovial fibroblasts transfected with WT or mutant MATN3 gene constructs. Quantification of GAG content by dimethylmethylene blue assay **(A)** and DNA quantification **(B)** of primary PSF pellet cultures. Normalizing GAG content to DNA content **(C)**. Each data point on the graph represents mean ±SDM from biological triplicate samples. Single asterisks ($p \leq 0.05$), double asterisks ($p \leq 0.01$) and triple asterisks ($p \leq 0.005$) indicate statistically significant differences from WT-MATN3 transfected primary PSFs. Statistical analysis was done using a one-way ANOVA followed by a Dunnett's test to determine whether differences between the WT-MATN3 group and other experimental groups are significant.

FIGURE 8

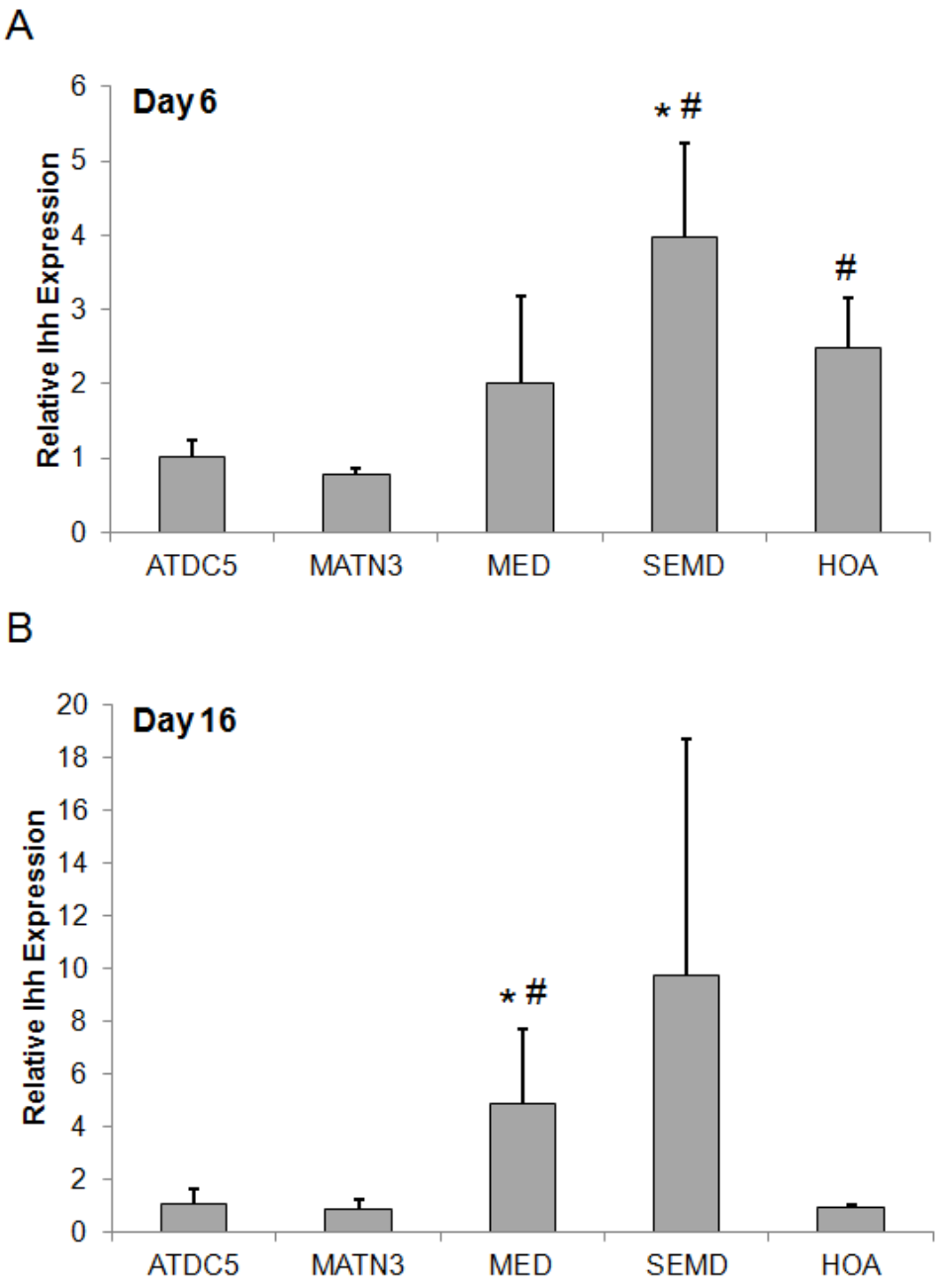

A
Day 6
Relative Ihh Expression
0
1
2
3
4
5
6
* #
#
ATDC5
MATN3
MED
SEMD
HOA
B
Day 16
Relative Ihh Expression
0
2
4
6
8
10
12
14
16
18
20
* #
ATDC5
MATN3
MED
SEMD
HOA

Figure 8: Indian hedgehog mRNA levels in ATDC5 chondroprogenitors stably transfected with WT or mutant MATN3 gene constructs. Relative mRNA expression levels of Indian hedgehog in MATN3 transfected ATDC5 lines 6 days **(A)** and 16 days **(B)** after monolayer culture. Experiments were done in biological triplicates. Single asterisks ($p \leq 0.05$) indicate statistically significant differences from untransfected parental ATDC5 cells. Hash marks ($p \leq 0.05$) indicate statistically significant differences from WT-MATN3 transfected ATDC5 cells.

FIGURE 9

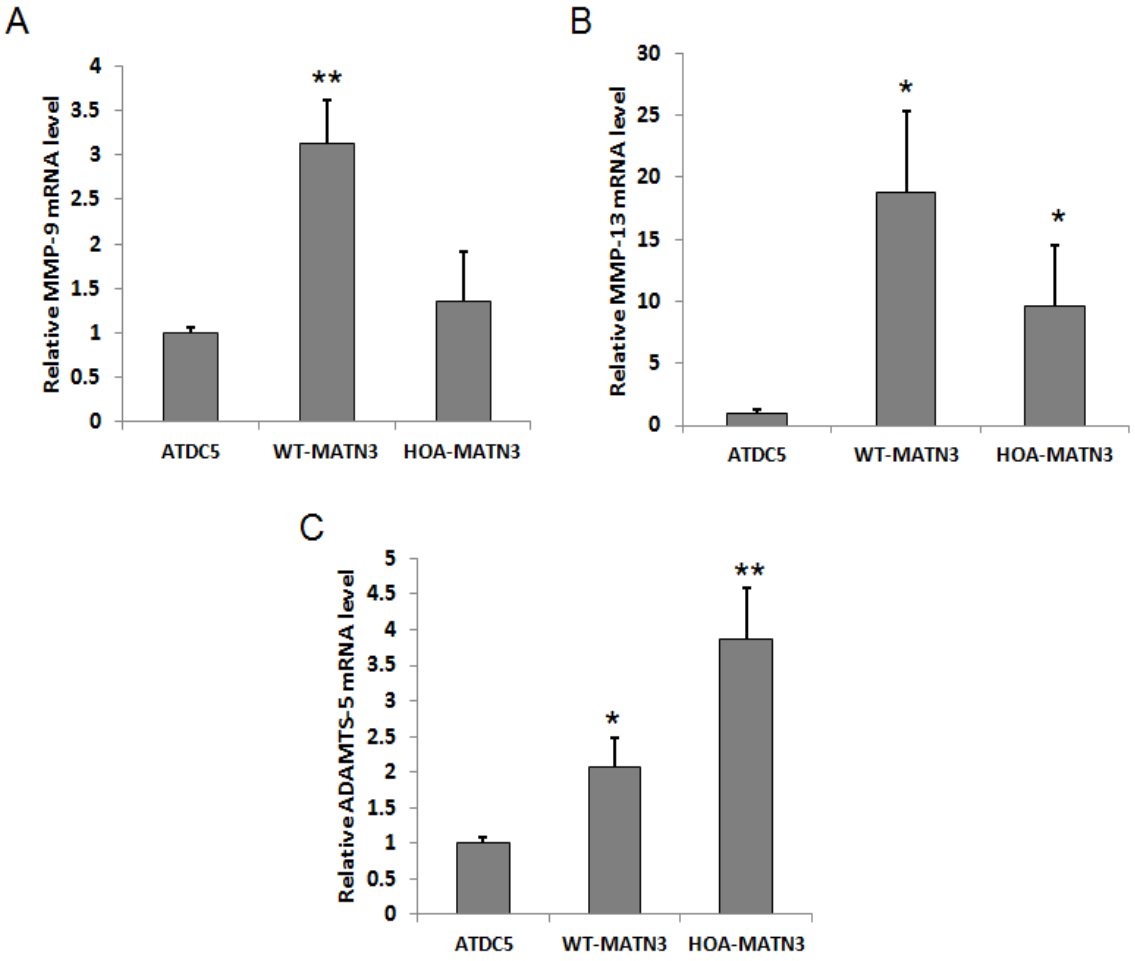

A
Relative MMP-9 mRNA level
4
3.5
3
2.5
2
1.5
1
0.5
0
**
ATDC5
WT-MATN3
HOA-MATN3
B
Relative MMP-13 mRNA level
30
25
20
15
10
5
0
*
*
ATDC5
WT-MATN3
HOA-MATN3
C
Relative ADAMTS-5 mRNA level
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
**
ATDC5
WT-MATN3
HOA-MATN3

Figure 9: Gene expression of cartilage resorption markers in WT or HOA MATN3 transfected ATDC5 chondroprogenitors. mRNA expression levels of MMP-9 (A) MMP-13 (B) and ADAMTS-5 (C) in ATDC5 murine chondroprogenitors stably transfected with the WT or HOA MATN3 gene construct. Expression was measured after 24 hours of monolayer culture. Experiments were done in biological triplicates. Single asterisks ($p \leq 0.05$) and double asterisks ($p \leq 0.01$) indicate statistically significant differences from untransfected parental ATDC5 cells.

FIGURE 10

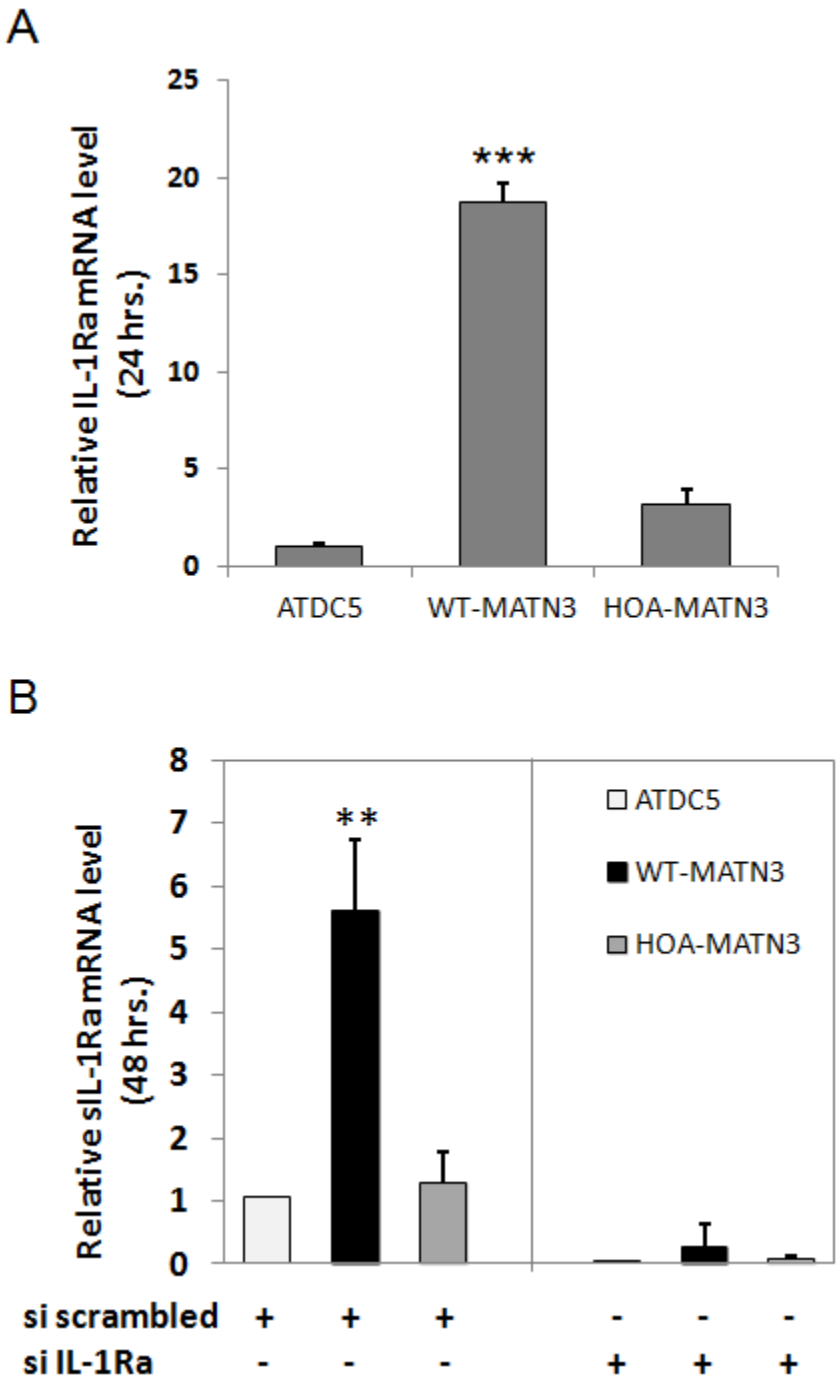

A
Relative IL-1Ra mRNA level (24 hrs.)
25
20
15
10
5
0
***
ATDC5
WT-MATN3
HOA-MATN3
B
Relative sIL-1Ra mRNA level (48 hrs.)
8
7
6
5
4
3
2
1
0
**
ATDC5
WT-MATN3
HOA-MATN3
si scrambled + + + - - -
si IL-1Ra - - - + + +

Figure 10. IL-1Ra levels are enhanced in MATN3 transfected ATDC5 chondroprogenitors. Relative mRNA expression of the anti-inflammatory cytokine IL-1Ra in MATN3 transfected and untransfected ATDC5 cells 24 hours after monolayer culture **(A)**. Treating transfected and untransfected ATDC5 cells with an siRNA against IL-1Ra caused an acute reduction of this gene in all tested cell lines **(B)**. Experiments were done in biological triplicates (for A) and quadruplicates (for B). Double asterisks ($p \leq 0.01$) and triple asterisks ($p \leq 0.005$) indicate statistically significant differences from untransfected parental ATDC5 cells.

FIGURE 11

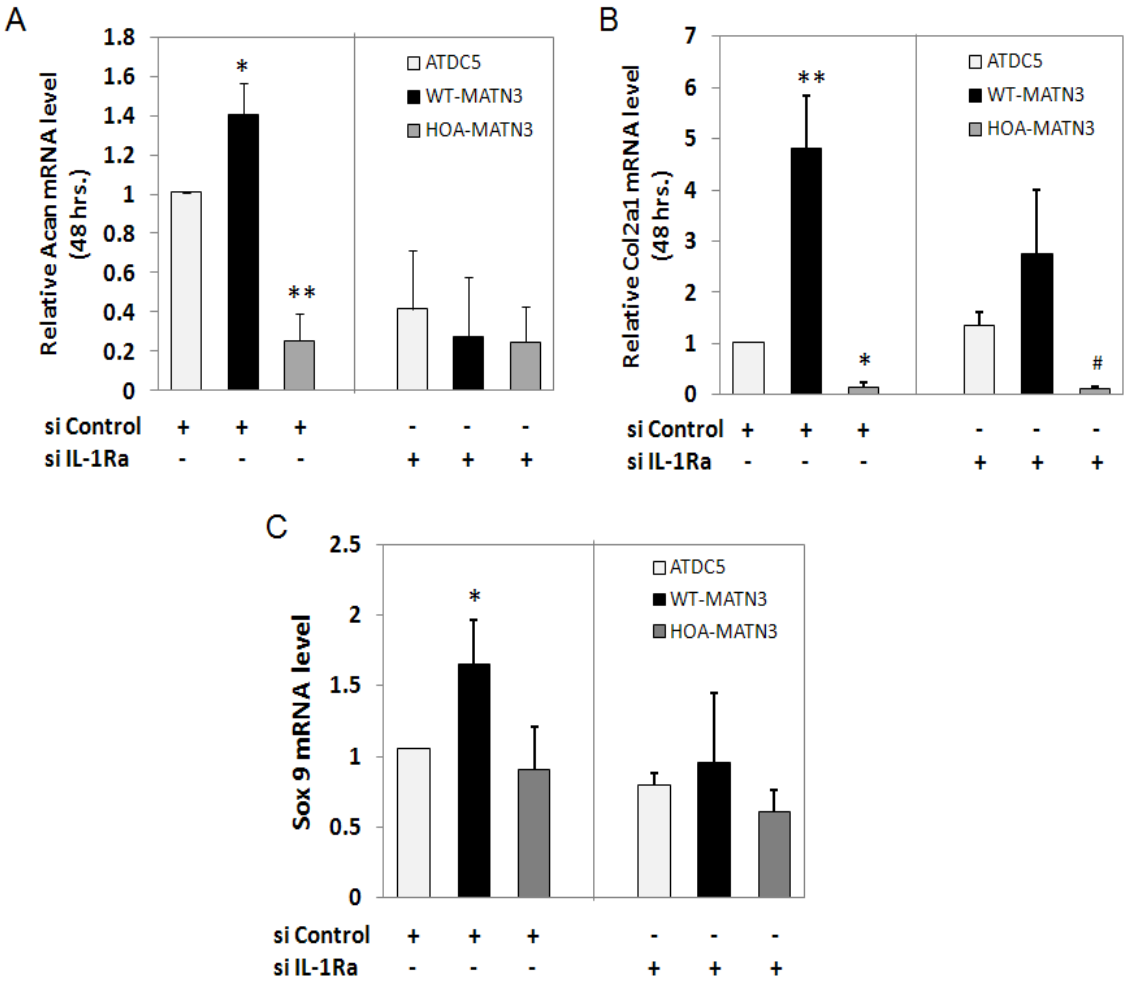

A
Relative Acan mRNA level (48 hrs.)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
ATDC5
WT-MATN3
HOA-MATN3
si Control + + + - - -
si IL-1Ra - - - + + +
B
Relative Col2a1 mRNA level (48 hrs.)
7
6
5
4
3
2
1
0
**
*
#
ATDC5
WT-MATN3
HOA-MATN3
si Control + + + - - -
si IL-1Ra - - - + + +
C
Sox 9 mRNA level
2.5
2
1.5
1
0.5
0
*
ATDC5
WT-MATN3
HOA-MATN3
si Control + + + - - -
si IL-1Ra - - - + + +

Figure 11: MATN3 induction of chondrogenesis markers before and after IL-1Ra knock down. Relative mRNA expression of aggrecan **(A)**, type II collagen **(B)** and sox9 **(C)** by ATDC5 cells that are untransfected or stably transfected with the WT or HOA MATN3 gene construct in the presence and absence of a siRNA against IL-1Ra. Single asterisks ($p \leq 0.05$) and double asterisks ($p \leq 0.01$) indicate statistically significant differences from parental ATDC5 cells treated with scrambled siRNA control. Hash marks ($p \leq 0.05$) indicate statistically significant differences from parental ATDC5 cells treated with siRNA against IL-1Ra.

FIGURE 12

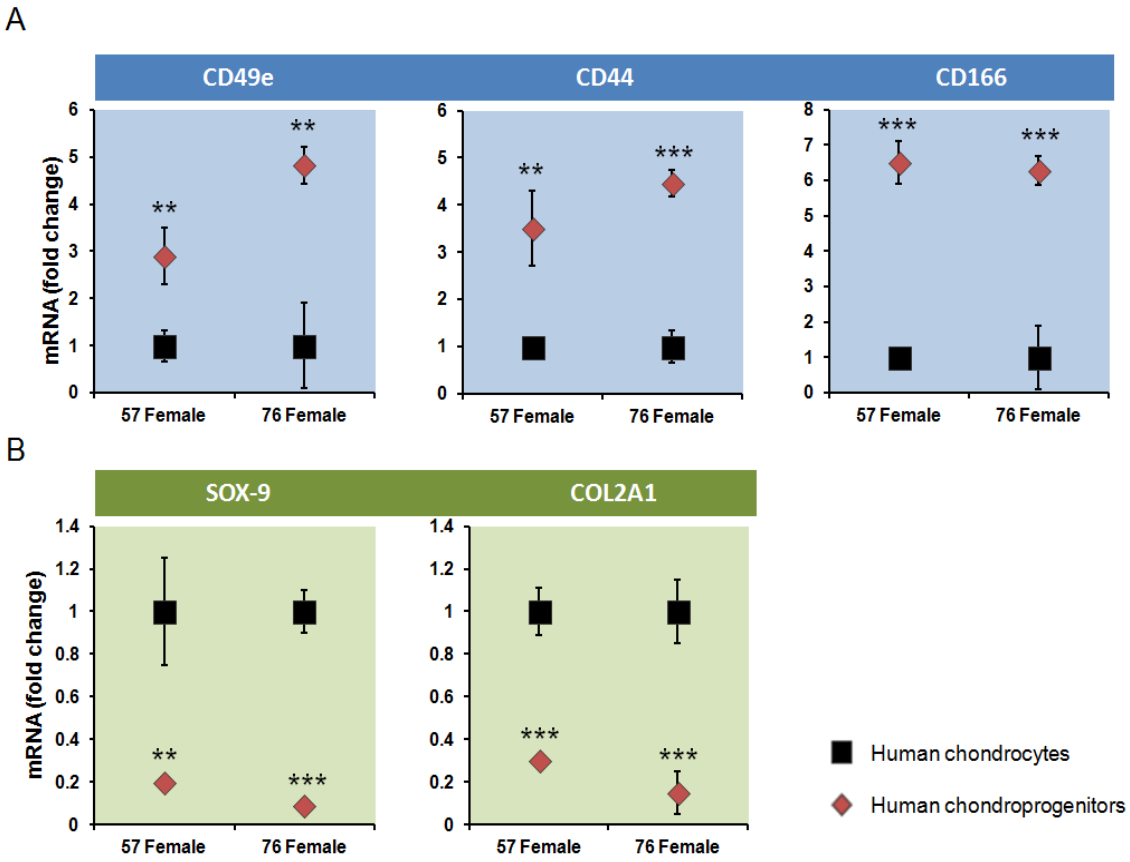

A
CD49e
CD44
CD166
mRNA (fold change)
57 Female
76 Female
B
SOX-9
COL2A1
Human chondrocytes
Human chondroprogenitors

Figure 12: Characterization of primary human chondroprogenitors isolated from patients. Fold change of mesenchymal stem cell markers CD49e, CD44 and CD166 in human chondroprogenitor cells isolated from two female patients that are 57 and 76 year of age, relative to their primary human chondrocytes (A). Fold change of chondrocyte markers SOX9 and type II collagen in the same patients, relative to their primary human chondrocytes (B). Experiments were done in biological triplicate. Double asterisks ($p \leq 0.01$) and triple asterisks ($p \leq 0.005$) indicate statistically significant differences from patient specific primary chondrocyte controls.

FIGURE 13

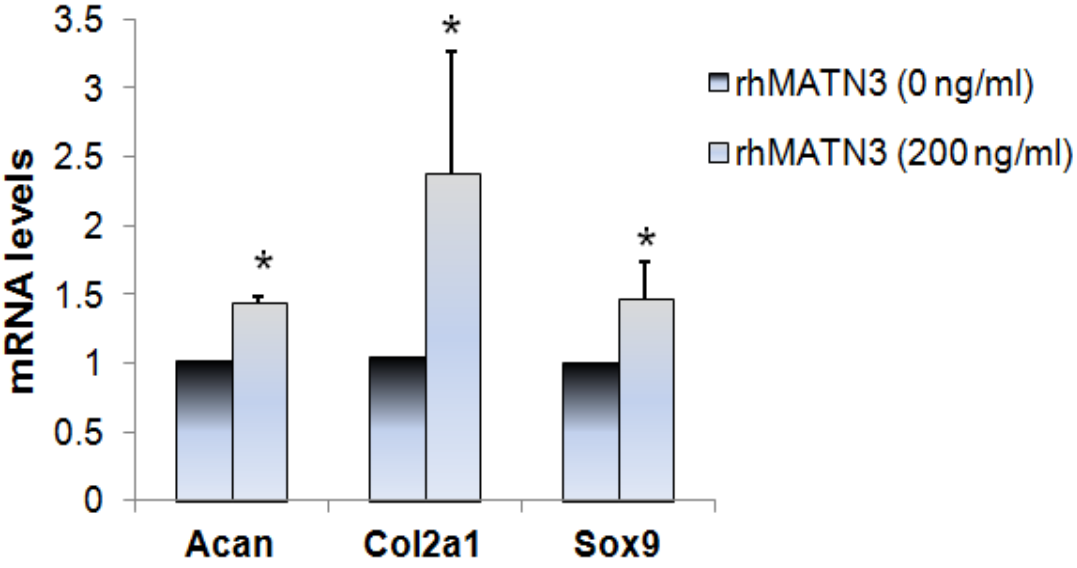

3.5
3
2.5
2
1.5
1
0.5
0
mRNA levels
*
*
*
Acan
Col2a1
Sox9
rhMATN3 (0 ng/ml)
rhMATN3 (200 ng/ml)

Figure 13: Recombinant human MATN3 stimulates chondrogenesis marker expression in primary human chondroprogenitors. Enhancement of chondrogenesis markers aggrecan, type II collagen and SOX9 was observed in primary human chondroprogenitor cells treated with recombinant human MATN3 protein for 24 hours. Experiments were done in biological triplicate. Asterisks ($p \leq 0.05$) indicate statistically significant differences from the untreated primary human chondroprogenitor controls.

FIGURE 14

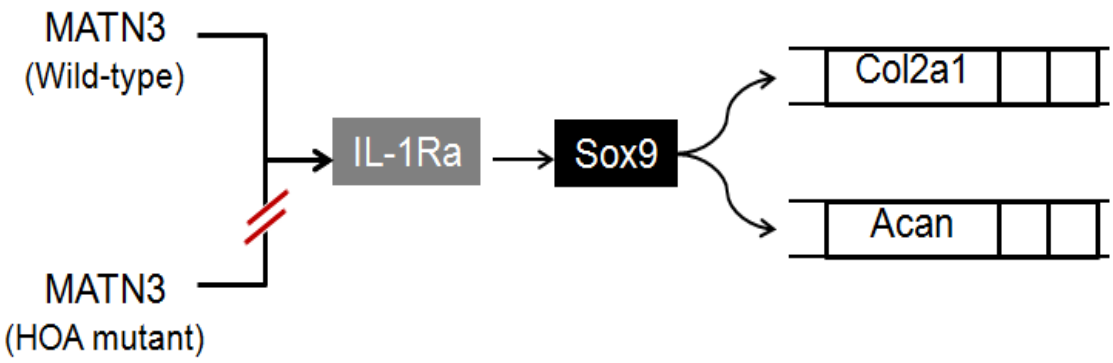


Figure 14: Proposed pathway: MATN3 regulates key chondrogenesis markers in an IL-1Ra dependent manner. WT-MATN3, but not HOA-MATN3, can stimulate IL-1Ra, which promotes chondrogenesis markers including the transcription factor Sox9 and downstream markers aggrecan and type II collagen in chondroprogenitor cells.

CHAPTER 4

AIM III

EGFR activity and PI3 kinase pathway signaling are required to mediate IL-1Ra-dependent regulation of cartilage homeostasis markers by MATN3

Manuscript in preparation

EGFR activity and PI3 kinase pathway signaling are required to mediate the IL-1Ra-dependent regulation of cartilage homeostasis markers by MATN3

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Keywords: Matrilin-3, IL-1Ra, EGFR, PI3 kinase, chondrocytes, signal transduction.

Abstract

Introduction: It has been previously demonstrated that matrilin-3 (MATN3) has several regulatory properties that go beyond its classically defined role as a bridging molecule for extracellular matrix (ECM) networks in articular cartilage tissue. The present study investigates how MATN3 affects chondrocyte cell signaling and how this may help explain IL-1 receptor antagonist (IL-1Ra) dependent and independent regulatory functions of MATN3.

Methods: C28/I2 immortalized human chondrocytes or primary human chondrocytes were treated with recombinant human (rh) MATN3 protein. Western blot analysis was used to test for rapid phosphorylation of Akt, GSK3, ERK1/2 and p38 in these cells. Inhibitor studies, using PI3 kinase and EGFR inhibitors, and MKK6 over-expression were used to determine whether the effects of MATN3 on downstream markers including IL-1Ra, type II collagen, aggrecan and ADAMTS-5 can be disregulated by altering these cell signaling pathways. Real-time PCR was utilized to measure expression of MATN3 downstream markers.

Results: rhMATN3 treatment of chondrocytes for 30 minutes resulted in phosphorylation of Akt, GSK3 and ERK1/2 and inhibited p38 phosphorylation. Inhibition of PI3 kinase altered MATN3's regulation of type II collagen and aggrecan. Inhibition of EGFR altered the regulation by MATN3 of IL-1Ra, type II collagen, aggrecan and ADAMTS-5. Constitutive activation of p38 via

overexpression of MKK6 in chondrocytes did not alter the regulation of MMP-13 by MATN3 in these cells.

Conclusion: MATN3 can directly initiate chondrocyte cell signaling. The effects of MATN3 on IL-1Ra and downstream cartilage homeostasis markers depend on EGFR activity and PI3 kinase pathway signaling. Although MATN3 inhibits phosphorylation of p38, its regulation of MMP-13 is independent of p38 activity.

Introduction

Articular cartilage is low in cell content compared to most other types of tissue in our bodies. It is mostly comprised of extracellular matrix (ECM) molecules produced by a sparse population of chondrocytes. The utility that many ECM molecules provide by maintaining the structural integrity, which is required to perform the primary function of load bearing in tissue such as cartilage, is undeniable. However, many cartilage ECM molecules also have roles that go beyond their structural utility [1-4]. Matrilin-3 (MATN3) is an example of such an ECM molecule. It is expressed by articular chondrocytes and highly expressed by growth plate chondrocytes during development [5, 6]. Its molecular structure consists of six protein domains, many of which have functions that are yet unknown. MATN3 has one Von Willebrand Factor A (vWFA) domain, four epidermal growth factor-like (EGF) domains, and an oligomerization domain that allows a MATN3 monomer to matriculate with one another to form trimers and tetramers [5]. MATN3 interacts with types II and type IX collagens [7] and also several non-collagenous ECM molecules [8]. Although MATNs are classically believed to play a purely structural role in cartilage tissue [8], in a recent study we identified several key cartilage tissue homeostasis markers that can be regulated by MATN3 in chondrocytes [3]. Although the exact role of this ECM protein is not fully understood, mutations in the MATN3 gene have been found to closely associate with several forms of chondrodysplasia [9-12] and hand osteoarthritis (HOA) [13, 14]. Moreover, the recently discovered ability of MATN3 to regulate the expression of cartilage homeostasis markers may explain why its functional

absence in murine cartilage tissue results in much earlier onset of osteoarthritis (OA) in these animals [6].

We have previously demonstrated that the regulation of anabolic markers type II collagen and aggrecan by MATN3 relies on its induction of the anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1Ra), whereas the inhibition effect of MATN3 on MMP-13 expression is completely independent of IL-1Ra [3]. IL-1Ra is a natural inhibitor of the IL-1β pathway [15-21]. It is an IL-1β ligand mimic that binds the IL-1 receptor and prevents the co-localization of this receptor with a transmembrane accessory protein that is required for initiating IL-1β pathway activation. IL-1Ra is produced by a variety of cells including articular chondrocytes and several studies suggest that it is protective against cartilage degradation in arthritis [18, 22-24].

Our previous study suggested that MATN3 utilizes both an IL-1Ra dependent and independent pathway to mediate specific regulatory effects in chondrocytes. Interestingly, several kinases that are involved in the induction of IL-1Ra in inflammatory cells have been previously identified [25-28] In the present study, we use human chondrocytes to test whether recombinant human MATN3 is capable of phosphorylating and activating these cell signaling molecules. We also show evidence strongly suggesting that the induction of IL-1Ra in chondrocytes is a direct effect of MATN3, and not a delayed secondary effect. Furthermore, we show that MATN3 can inhibit activation of p38, which is a MAP kinase that is upstream of MMP-13 [29, 30]. Finally, we utilize PI3 kinase and epidermal growth factor receptor (EGFR1) inhibitor studies, as well as a p38

overexpression, to test whether these molecules play essential roles in MATN3 signaling in articular chondrocytes.

Materials and methods

DMEM and fetal bovine serum (FBS) used for cell culture were purchased from Invitrogen (Carlsbad, CA, USA). The following primary antibodies for Western blot analysis were purchased from Cell Signaling (Danvers, MA, USA): Phospho-Akt T308 mAb, Akt Ab, Phospho-GSK-3beta S9 mAb, GSK-3beta mAb, Phospho-ERK1/2 mAb, ERK1/2 mAb, Phospho-p38 MAPK Thr180/Tyr182 mAb, p38 MAPK mAb. The C28/I2 human chondrocyte cell line was a generous gift from Dr. Mary Goldring. The pcDNA3-Flag MKK6 (glu) vector construct was a generous gift from Dr. Roger Davis.

Isolation of primary human chondrocytes

As described in Chapter 2, primary human chondrocytes (PHCs) were isolated from normal knee articular cartilage obtained from patients undergoing total joint replacement surgery. Primary chondrocytes were isolated from all zones of patient cartilage. Samples were handled independently without pooling and processed separately to isolate chondrocytes. Shortly after tissue collection, patient cartilage samples were washed twice with sterile PBS and cut into small fragments. These tissue fragments were digested in 5.0 mL of Pronase (Roche, Indianapolis, IN, USA) in HBSS at a concentration of 2.0 mg/mL for 30 minutes at 37 °C under shaking conditions. Then the pronase digestion solution was

removed and cartilage fragments were washed twice with DMEM (Life Technologies, Grand Island, NY, USA). DMEM was then removed and replaced with 5.0 mL of Type IA Crude Bacterial Collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 1.0 mg/mL and incubated for 8 hours at 37°C under shaking conditions. 5.0 mL of DMEM containing 10% FBS was added into the digestion mix to halt the collagenase enzymatic reaction. Undigested tissue clumps were removed from the solution by straining through a 100-µm nylon mesh cell strainer (BD, Franklin Lakes, NJ, USA). The strained solution was then centrifuged at 1500 rpm to pellet the successfully isolated chondrocytes. The resulting cell pellet was washed twice with DMEM and cells were counted using Trypan Blue (BioWhittaker, Walkersville, MD, USA) and a hemocytometer. Chondrocytes were plated at high density (4.0×10^6 cells) in 60 mm cell culture dishes using DMEM supplemented with 10% FBS and 0.2% Streptomycin (Life Technologies, Grand Island, NY, USA). All studies involving patient tissue samples were conducted in accordance with the Institutional Review Board (IRB) of Rhode Island Hospital. Patient consent for collection of cartilage tissue was waived by the IRB pf Rhode Island Hospital.

Experimental cell culture conditions

In this study, cell culture experiments were conducted using either C28/I2 human chondrocytes or primary human chondrocytes. Cells were seeded at a density of 200,000 cells per well in 6-well culture plates. DMEM supplemented with 10% FBS and 0.2% streptomycin was used as culture medium.

Recombinant human MATN3 protein (R&D Systems, Minneapolis, MN) was used at a concentration of 200 ng/ml and recombinant human IL-1β protein (PeproTech, Rocky Hill, NJ) was used at a concentration of 5.0 ng/ml in experiments. Cells were treated with these recombinant proteins for 30, 60, 120 minutes or 24 - 48 hours depending on the experiment. See figure legends for further details.

PI3 kinase and EGFR inhibitor treatment of chondrocytes

For PI3 kinase inhibition experiments, C28/I2 human chondrocytes were first seeded at 200,000 cells per well in 6-well culture dishes in DMEM supplemented with 10% FBS. After 24 hours, cell culture medium was removed and replaced with unsupplemented DMEM and the incubation was continued for 5 hours. After serum starvation, the cells were treated with the PI3 kinase chemical inhibiter Ly294002 (Cell Signaling, Danvers, MA, USA) at 10 μM concentration in the presence or absence of 200 ng/ml or recombinant human MATN3 protein for 24 hours in DMEM. Control C28/I2 cells were grown for 24 hours in DMEM, in the presence or absence of MATN3, without PI3 kinase inhibitor.

Similarly, EGFR inhibition experiments were conducted by first plating the same density of C28/I2 cells in 6 well culture dishes followed by 24 hours of growth in DMEM supplemented with 10% FBS. Cells were then serum starved for 5 hours and treated with DMEM containing the EGFR inhibitor AG494 (Sigma-Aldrich, St. Louis, MO, USA) at 10 μM in the presence or absence of the

200 ng/ml or recombinant human MATN3 protein for 24, as this concentration of MATN3 was previously shown as sufficient to mediate regulation of chondrocyte homeostasis genes [3]. As before, control cells were grown for 24 hours in DMEM in the presence or absence of MATN3, without EGFR inhibitor.

Transient transfection of primary human chondrocytes

Primary human articular chondrocytes were grown in monolayer culture in 60 mm cell culture dishes using DMEM supplemented with 10% FBS and 0.2% streptomycin (Life Technologies, Grand Island, NY, USA) until 95% confluent. An 8.0 ml solution of DMEM containing 1.0 mg/ml Pronase (Roche, Indianapolis, IN, USA) and 1.0 mg/ml Type IA Crude Bacterial Collagenase (Sigma-Aldrich, St. Louis, MO, USA) was added to the chondrocyte culture to help break up ECM networks to free cells making them more susceptible to transfection via electroporation. Cells were incubated with this pronase/collagenase solution for 4 hours at 37°C, collected and centrifuged at 1500 RPM for 5 minutes. The cell pellet was washed twice with DMEM to remove residual enzyme solution. Cells were counted using Trypan Blue (BioWhittaker, Walkersville, MD, USA) and a hemocytometer. An Amaxa Nucleofector kit (Lonza) was used to transiently transfect cells according to the manufacturer's instructions. Each transfection reaction used 500,000 cells and 2.0 µg of pcDNA3-Flag MKK6 (glu) vector. Cells in control groups were transfected with 2.0 µg of empty pcDNA3 vector. To ensure both transfection efficiency and transfection longevity was optimal when using the Amaxa Nucleofector to transfect primary human chondrocytes, 2.0 µg

of a GFP construct (provided by manufacturer) was transfected into 500,000 chondrocytes. The transfection efficiency was approximately 70-75% with the GFP signal fading no earlier than 96 hours post transfection (data not shown).

RNA isolation and Real-time PCR

Gene expression analysis was conducted by first isolating the total RNA from cell lysates using the RNAqueous Kit (Ambion, Austin, TX, USA) according to manufacturer's instructions. To make a cDNA template for the purpose of conducting real-time polymerase chain reaction (PCR), 0.5 ug of RNA from each sample was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Real-time PCR was conducted using DNA Engine Opticon 2 (Bio-Rad, Hercules, CA, USA) and the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA). As indicated in Table 1, specific primer pairs for human genes encoding IL-1Ra, type II collagen, aggrecan, MMP-13 and ADAMTS-5 were used. Relative mRNA transcript levels were calculated using the standard delta delta Ct ($\Delta\Delta$Ct) method, normalized to rRNA 18S expression according to the following equation: $X = 2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = (Ct_{Exp} - Ct_{18S}) - (Ct_{Ctl} - Ct_{18S})$ and X = Relative transcript; Ct_{Ctl} = Ct of control group.

Western blot analysis

Chondrocyte cell pellets were resuspended in 50 µl of 1x RIPA buffer (Cell Signaling Technology, Boston, MA, USA) containing protease inhibitors. Bradford

Assay (Bio-Rad, Herculese, CA, USA) and a NanoDrop 2000c (Thermo Fisher Scientific, Rockford, IL, USA) was used to quantify protein, according to the manufacturer's instructions. Proteins were transferred onto nitrocellulose membrane (Bio-Rad, Herculese, CA, USA) via a standard Western blot procedure. The membrane was blocked for 1 hour with 2% bovine serum albumin (BSA) in 1x tris buffer saline containing 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA)(TBST). The membrane was washed 3 times in TBST and incubated for 3 hours with primary antibody followed by 3 more washes with TBST. Finally the membrane was incubated for 1 hour with IRDye® 800CW Goat anti-Rabbit IgG (LI-COR Biosciences, Lincoln, Nebraska USA) at 1:10,000 dilution. The membrane was imaged using the Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE, USA). Band intensities were quantified using ImageJ software program (NIH, Bethesda, MD).

Statistical analysis

Mean values were calculated and presented with error bars representing ±SDM (one standard deviation of the mean). Two-tailed t-tests were used to analyze data represented in Figures 2B, 2C, 3B, 3C, 3D and 3E. In other cases, statistical analysis was done using a one-way ANOVA followed by post hoc test analysis. Statistical significance was considered at $P < 0.05$ for all analyses.

Results

MATN3 treatment of cells induces rapid phosphorylation of PI3 kinase pathway members involved in IL-1Ra stimulation

The molecular pathway(s) leading to IL-1Ra expression by cells are not yet fully understood. However, a previous study has demonstrated that IL-1Ra induction requires the phosphorylation of several downstream members of the PI3 kinase signaling pathway, including Akt, GSK3 and ERK-MAP kinase in innate immune cells [28]. To determine whether MATN3 can directly modulate known IL-1Ra pathway members, primary human chondrocytes were exposed to serum-free DMEM supplemented with only 200ng/ml of recombinant human MATN3 (rhMATN3) protein during a time course of 0, 30, 60 and 120 minutes. Western blot analysis showed marked elevation (35-fold) in phospho-Akt in chondrocytes treated with rhMATN3 at the earliest tested time point of 30 minutes (Figure 1A). Phospho-Akt levels continued to increase (43-fold) at 60 minutes, and finally peaked (60-fold) at the last tested time point of 120 minutes. Phosphorylation of GSK3 was also observed in MATN3-treated chondrocytes (Figure 1B). Phospho-GSK3 levels began to become noticeably elevated (1.5 fold) at the 60-minute interval and continued to increase to 2-fold by 120 minutes. Similarly, MATN3 treatment increased the phosphorylation of ERK1/2 (Figure 1C). A 2 fold elevation of phospho-ERK1/2 was evident as early as 30 minutes after MATN3 treatment. The phospho-ERK1/2 band intensity continued to increase to 4-fold at 60 minutes, where it peaked, and declined to 3-fold by the last tested time point of 120 minutes.

PI3 kinase activity is required to maintain MATN3 stimulation of type II collagen and aggrecan

In our previous study, we demonstrated that MATN3 can stimulate anabolic markers type II collagen (COL2A1) and aggrecan (ACAN) in chondrocytes in an IL-1Ra dependent manner [3]. To test whether activation of the PI3 kinase pathway is indeed a critical prelude to the regulation by MATN3 of cartilage homeostasis markers that lie downstream of IL-1Ra, we inhibited activation of PI3 kinase in chondrocytes using a chemical inhibitor and then observed the resulting effect on the ability of MATN3 to induce COL2A1 and ACAN in these cells. In the absence of the chemical inhibitor, MATN3 treatment for 60 minutes resulted in phosphorylation and activation of PI3 kinase in primary human articular chondrocytes (Figure 2A). However, treating cells with the chemical inhibitor, Ly294002, not only abolished the MATN3-induced phosphorylation of PI3 kinase, but also eliminated the basal phosphorylation of this kinase. Inhibitor treatment did not affect total PI3 kinase protein levels nor did it affect cell viability (data not shown).

Next, the mRNA expression of COL2A1 (Figure 2B) and ACAN (Figure 2C) were measured in these PI3 kinase inhibited cells using real-time PCR analysis. In the absence of PI3 kinase inhibition, MATN3 treatment for 24 hours enhanced expression of COL2A1 in chondrocytes by approximately 1.8-fold (Figure 2B, left). However, MATN3 could not significantly elevate COL2A1 expression in chondrocytes that were treated with PI3 kinase inhibitor (Figure 2B, right). The same trend was observed when looking at the induction of ACAN by

MATN3 in chondrocytes. MATN3 treatment enhanced ACAN mRNA expression levels by approximately 3.3-fold in the absence of PI3 kinase inhibition (Figure 2C, left), whereas it was unable to significantly elevate ACAN expression when chondrocytes were treated with the PI3 kinase inhibitor (Figure 2C, right).

EGFR activity is required for MATN3 induction of IL-1Ra and downstream cartilage homeostasis markers

The aforementioned findings demonstrate that exposure of chondrocytes to MATN3 protein results in rapid activation of PI3 kinase pathway elements including Akt, GSK3 and ERK. This suggests that MATN3 is capable of directly regulating chondrocyte cell signaling. To elucidate the biological mechanism that may allow MATN3 to initiate signal transduction in chondrocytes, we decided to first look at its basic protein structure for answers (Figure 3A). Since several known MATN3 mutations that severely hinder or alter its function occur within the EGF-like domains of MATN3 [12, 13], we decided to investigate whether MATN3 signals through EGFR in chondrocytes.

We measured the capacity of MATN3 to induce IL-1Ra and several downstream cartilage homeostasis markers, in chondrocytes that were treated or left untreated with the EGFR inhibitor AG494. MATN3 induction of IL-1Ra was evident in chondrocytes that were not treated with EGFR inhibitor; however, chemical inhibition of EGFR via AG494 not only prevented MATN3 induction of IL-1Ra, but also reduced basal expression of IL-1Ra (Figure 3B). Similarly, MATN3 treatment elevated COL2A1 mRNA levels in chondrocytes in the

absence of AG494, while it had no noticeable effect on COL2A1 expression in chondrocytes that were treated with AG494 (Figure 3C). Furthermore, AG494 treatment of chondrocytes also hindered MATN3's ability to elevate ACAN mRNA levels in a statistically significant manner (Figure 3D). Finally, inhibition of the aggrecanase ADAMTS-5 by MATN3 was not evident in chondrocytes treated with AG494 (Figure 3E).

MATN3 inhibits p38 MAP kinase activity in primary human chondrocytes

In our previous study, we showed that MATN3 can regulate MMP-13 independently of IL-1Ra [3]. However, the exact biological pathway responsible for this effect remains unknown. It has been previously established that p38 MAP kinase is upstream of MMP-13 expression in chondrocytes [29, 30]. Since aforementioned findings demonstrate that MATN3 treatment results in rapid activation of several key cell signaling molecules, we wanted to determine if MATN3 treatment can also affect p38 activity. For this purpose, primary human chondrocytes were treated with 200 ng/ml of rhMATN3 for 0, 30, 60 or 120 minutes. Western blot analysis was used to detect phospho-p38 and total p38 (Figure 4A). Chondrocytes treated with 5.0 ng/ml of IL-1β were used as a positive control for p38 phosphorylation/activation. MATN3 treatment initially induced transient phosphorylation of p38 at 30 minutes; however, by 60 minutes the phospho-p38 levels declined below baseline (Figure 4B). This pattern of p38 inhibition by MATN3 was also evident at the 120 minute interval.

Regulation of MMP-13 by MATN3 is independent of p38 MAP kinase

To test whether the inhibition by MATN3 of p38 MAP kinase activity is required for its regulation of MMP-13 expression, we transiently transfected primary human chondrocytes with the pcDNA3-Flag MKK6 (glu) expression vector, which carries a MKK6 transgene (Figure 5A). This vector has been shown to constitutively activate p38 [31]. Western blot analysis of cell lysates, 8 hours post transfection, indicated that p38 activity was significantly higher in chondrocytes transfected with the pcDNA3-Flag MKK6 (glu) construct relative to chondrocytes transfected with an empty pcDNA3 vector control (Figure 5B).

Finally, chondrocytes transfected with pcDNA3-Flag MKK6 (glu) and chondrocytes transfected with the empty pcDNA3 vector were treated with rhMATN3 protein. After MATN3 treatment for 72 hours, MMP-13 expression was analyzed using real-time PCR (Figure 5C). IL-1β treatment was used as a positive control for MMP-13 expression. MATN3 treatment induced downregulation of MMP-13 mRNA levels in both pcDNA3-transfected controls and pcDNA3-Flag MKK6 (glu)-transfected cells. Constitutive activation of p38 by MKK6 overexpression did not appear to affect the ability of MATN3 to suppress MMP-13 mRNA levels.

Discussion

It was once hypothesized that the primary role of cartilage specific matrix protein MATN3 is to bridge gaps between macromolecular components of the

cartilage ECM helping these networks interconnect thereby maintaining structural integrity of the tissue space [8]. Today, it is clear that the functional significance of MATN3 goes well beyond that of a structural protein. Recent findings suggest that MATN3 is involved in a wide range of developmental and cellular processes including chondrogenic differentiation [4], homeostatic balance and anti-inflammation [3, 32]. In the present study we have elucidated the cellular mechanisms underlying the IL-1Ra-dependent and independent regulation by MATN3 of various cartilage homeostasis markers including type II collagen, aggrecan, ADAMTS-5 and MMP-13.

We demonstrate that MATN3 treatment of chondrocytes for only 30 minutes results in phosphorylation of multiple signal transduction mediators. This discovery strongly suggests that MATN3 can directly activate chondrocyte cell signaling. Specifically, MATN3 is capable of activating the PI3 kinase pathway. Since previous studies have shown that PI3 kinase signaling is required for IL-1Ra induction in other cell types [25, 27, 28], it is possible that MATN3 stimulation of IL-1Ra may also be mediated through this pathway. Moreover, we have demonstrated that chemically inhibiting PI3 kinase activity completely inhibits the IL-1Ra dependent induction of type II collagen and aggrecan by MATN3. This further supports the hypothesis that PI3 kinase activity is necessary for the induction by MATN3 of IL-1Ra and downstream markers of cartilage anabolism.

Interestingly, the molecular structure of MATN3 includes several EGF-like repeats so named because they share a common sequence of up to 50 amino

acids with EGFR ligands. Mutations in the first EGF-like repeat of MATN3 are closely associated with chonrodysplasia and HOA [9-12]. Since we have shown here that MATN3 is capable of initiating chondrocyte cell signaling, it is plausible that this EGF-like domain is necessary to stimulate such cellular responses. While the functional significance of EGF-like repeats in cartilage ECM proteins are currently unclear, some ECM molecules containing such repeats, like tenascin C, exhibit low binding affinity for EGFR [33, 34]. Moreover, loose binding of the 14th EGF-like repeat of tenascin C to EGFR results in altered biochemical and cellular responses compared to EGF itself [33], suggesting that EGFR signaling may vary based on ligand affinity. Furthermore, it is known that EGFR activation can result in IL-1Ra induction [35]. Here we have discovered that EGFR activity is also necessary to mediate the IL-1Ra-dependent regulation by MATN3 of the cartilage homeostasis markers, type II collagen, aggrecan, and ADAMTS-5 expression. Overall, these findings support the hypothesis that MATN3 signals through EGFR to regulate downstream cartilage homeostasis markers (Figure 6).

Unlike other tested homeostasis markers, we previously showed that MATN3 inhibits IL-1β-induced MMP-13 expression in an IL-1Ra independent manner [3]. Studies have shown that inhibiting p38, but not ERK, activity can downregulate IL-1β-induced MMP-13 expression suggesting that phosphorylation of p38 is a preamble to MMP-13 release in articular chondrocytes [36]. In the present study we have discovered that MATN3 can inhibit phosphorylation and activation of p38 in primary human chondrocytes. We tested whether MATN3's

inhibition of p38 activity is sufficient to account for its ability to inhibit MMP-13 by constitutively activating p38. We transfected primary human chondrocytes with a vector construct that leads to overexpression of MKK6, which constitutively activates p38 [31, 37]. Remarkably, while constitutive activation of p38 was observed in chondrocytes overexpressing MKK6, the elevation in p38 activity did not hinder the capacity of MATN3 to inhibit MMP-13 in these cells. This suggests that MATN3 does not depend on hindering p38 activity as a means of regulating MMP-13 expression in chondrocytes.

Conclusion

The present study demonstrates that cartilage specific ECM protein MATN3 is capable of quickly initiating cell signaling in chondrocytes suggesting that it can directly regulate cellular processes. This includes its activation of PI3 kinase pathway members, which previous studies have shown to be required for IL-1Ra induction. Moreover, we have demonstrated that disruption of EGFR and PI3 kinase activity can disrupt the regulation by MATN3 of downstream cartilage homeostasis markers. Finally, we show that MATN3 treatment of chondrocytes results in inhibition of p38 MAP kinase activity. However, this inhibition does not account for the effects of MATN3 on expression of the catabolic protease MMP-13.

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TABLE 1

Species	Gene	Forward sequence	Reverse sequence
Human	ADAMTS-5	5’-GGCCGTGGTGAAGGTGGTGG-3’	5’-GCTGCGTGGAGGCCATCGTC-3’
Human	ACAN	5’-ACCAGACGGGCCTCCCAGAC-3’	5’-TGGCTCTGCCCCAGAGGGAC-3’
Human	COL21A	5’-TGAGGGCGCGGTAGAGACCC-3’	5’-TGCACACAGCTGCCAGCCTC-3’
Human	IL-1Ra	5’-CCCGTGAAGGAGAGCCCTTCATTTG-3’	5’-ACTTTCACCATCATTTCACAAATGCAG-3’
Human	MMP-13	5’-ATGCGGGGTTCCTGATGTGG-3’	5’-GGCCCAGGAGGAAAAGCATG-3’

Table 1. Forward and reverse primer sequences used for Real-Time PCR

FIGURE 1

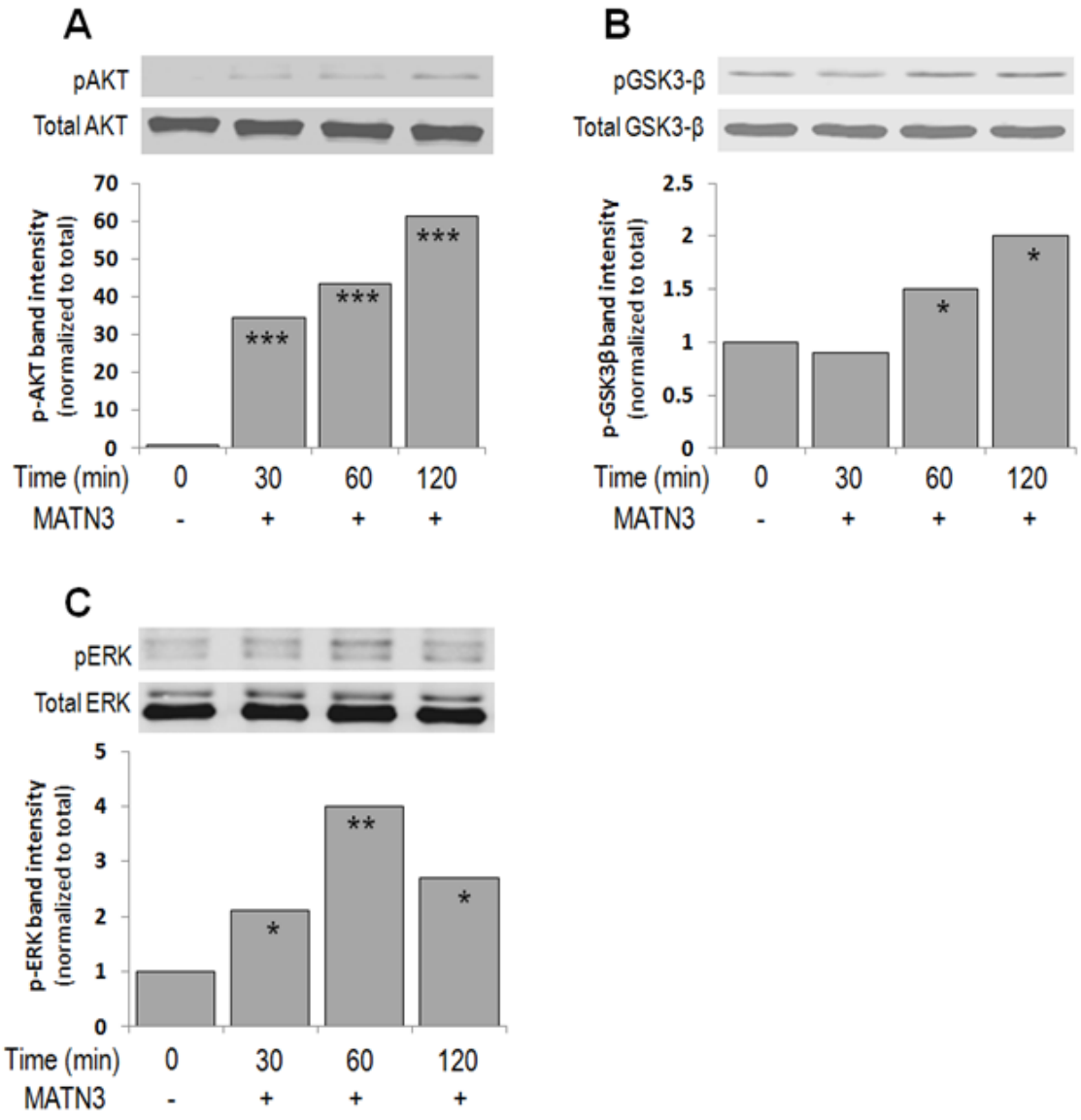

A
pAKT
Total AKT
p-AKT band intensity (normalized to total)
70
60
50
40
30
20
10
0
***
***
***
Time (min) 0 30 60 120
MATN3 - + + +
B
pGSK3-β
Total GSK3-β
p-GSK3β band intensity (normalized to total)
2.5
2
1.5
1
0.5
0
*
*
*
Time (min) 0 30 60 120
MATN3 - + + +
C
pERK
Total ERK
p-ERK band intensity (normalized to total)
5
4
3
2
1
0
*
**
*
Time (min) 0 30 60 120
MATN3 - + + +

Figure 1. MATN3 stimulates phosphorylation of several PI3 kinase pathway members. Treatment of C28/I2 human chondrocytes in a 0 to 120 minute time course with recombinant human MATN3 protein at a concentration of 200 ng/ml induces rapid phosphorylation of downstream members of the PI3 kinase pathway including Akt **(A)**, GSK3 **(B)** and ERK1/2 as analyzed by western blot **(C)**. Phospho-protein band intensities have been quantified and normalized to total protein band intensities below each western blot panel using ImageJ software program. Single asterisks ($p \leq 0.05$), double asterisks ($p \leq 0.01$) and triple asterisks ($p \leq 0.005$) indicate statistically significant differences from the untreated control group. Data are representative of 3 individual experiments.

FIGURE 2

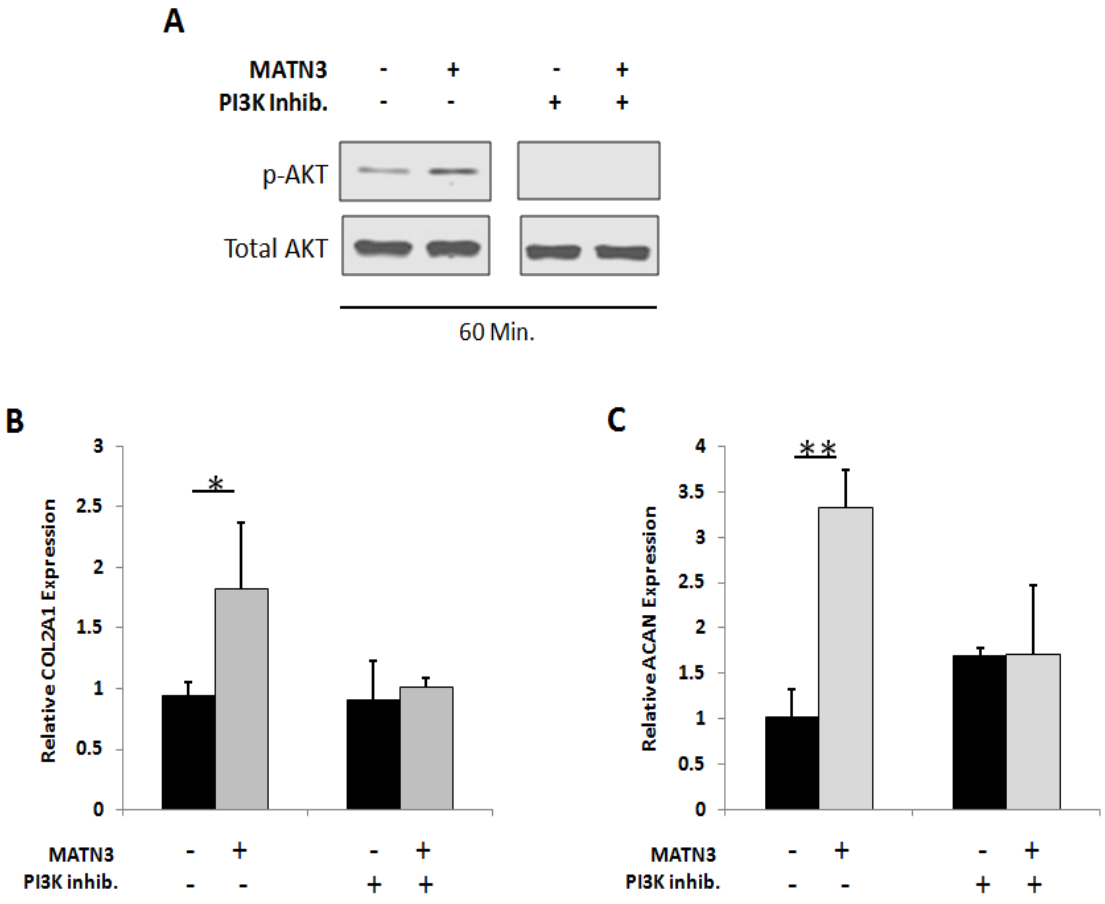

A
MATN3 - + - +
PI3K Inhib. - - + +
p-AKT
Total AKT
60 Min.
B
Relative COL2A1 Expression
3
2.5
2
1.5
1
0.5
0
*
MATN3 - + - +
PI3K inhib. - - + +
C
Relative ACAN Expression
4
3.5
3
2.5
2
1.5
1
0.5
0
**
MATN3 - + - +
PI3K inhib. - - + +

Figure 2. MATN3 regulation of type II collagen and aggrecan requires activation of PI3 kinase. Western blot analysis of phospho-Akt and total Akt band intensities in C28/I2 human chondrocytes treated with recombinant human MATN3 protein for 60 minutes, in the presence or absence of PI3 kinase inhibitor Ly294002 **(A)**. Real-time PCR quantification of type II collagen **(B)** and aggrecan gene expression in C28/I2 cells after 24 hours of treatment with recombinant MATN3 protein in the presence or absence of Ly294002 **(C)**. MATN3 protein was used at a concentration of 200 ng/ml for all experiments. PI3 kinase inhibitor Ly294002 was used at 10 μM for all experiments. Single asterisks ($p \leq 0.05$) and double asterisks ($p \leq 0.01$) indicate statistically significant differences between indicated groups. Data are representative of 3 individual experiments.

FIGURE 3

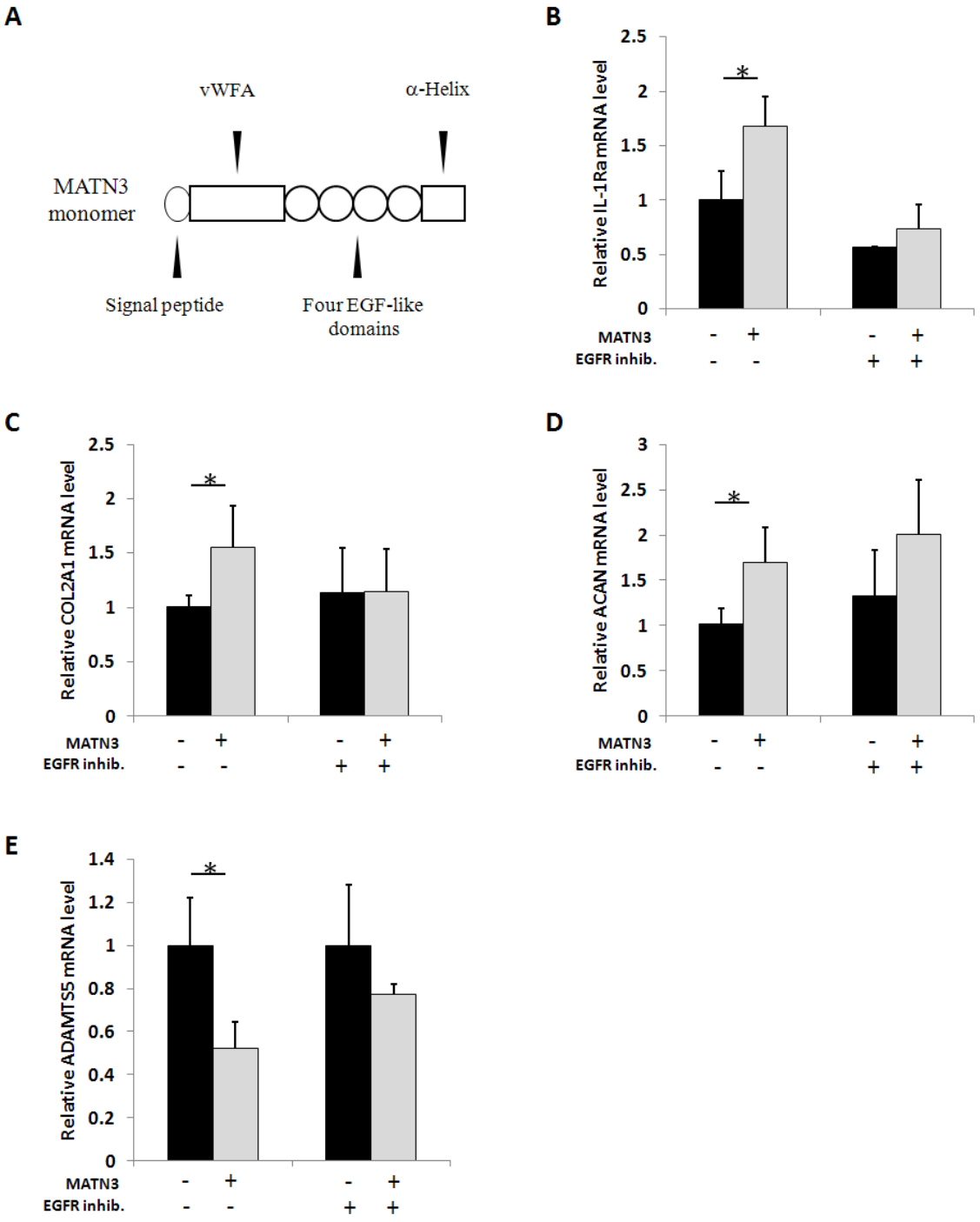
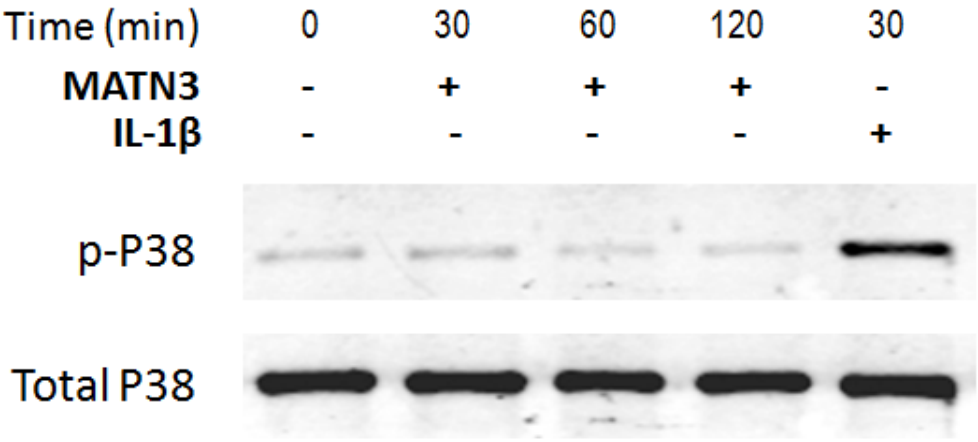

A
vWFA
α-Helix
MATN3
monomer
Signal peptide
Four EGF-like
domains
B
Relative IL-1Ra mRNA level
2.5
2
1.5
1
0.5
0
*
MATN3 - + - +
EGFR inhib. - - + +
C
Relative COL2A1 mRNA level
2.5
2
1.5
1
0.5
0
*
MATN3 - + - +
EGFR inhib. - - + +
D
Relative ACAN mRNA level
3
2.5
2
1.5
1
0.5
0
*
MATN3 - + - +
EGFR inhib. - - + +
E
Relative ADAMTS5 mRNA level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
MATN3 - + - +
EGFR inhib. - - + +

Figure 3. Inhibition of EGFR activity hinders MATN3 induced regulation of several key cartilage homeostasis markers. The basic protein structure of a MATN3 monomer contains a single signaling peptide, a single von willebrand factor A domain, 4 EGF-like domains and a single alpha-helical tail **(A)**. Real-time PCR quantification of IL-1Ra as stimulated by recombinant MATN3 and IL-1β treatment in the presence or absence of EGFR inhibitor AG494 **(B)**. Real-time PCR quantification of type II collagen **(C)**, aggrecan **(D)** and ADAMTS-5 gene expression in the presence or absence of AG494 **(E)**. C28/I2 human chondrocytes were used for all experiments. MATN3 protein was used at a concentration of 200 ng/ml, IL-1β was used at 5.0 ng/ml and EGFR inhibitor AG494 was used at 10 μM. Single asterisks ($p \leq 0.05$) indicate statistically significant differences between indicated groups. Data are representative of 3 individual experiments.

FIGURE 4

A


Time (min) 0 30 60 120 30
MATN3 - + + + -
IL-1β - - - - +
p-P38
Total P38

B

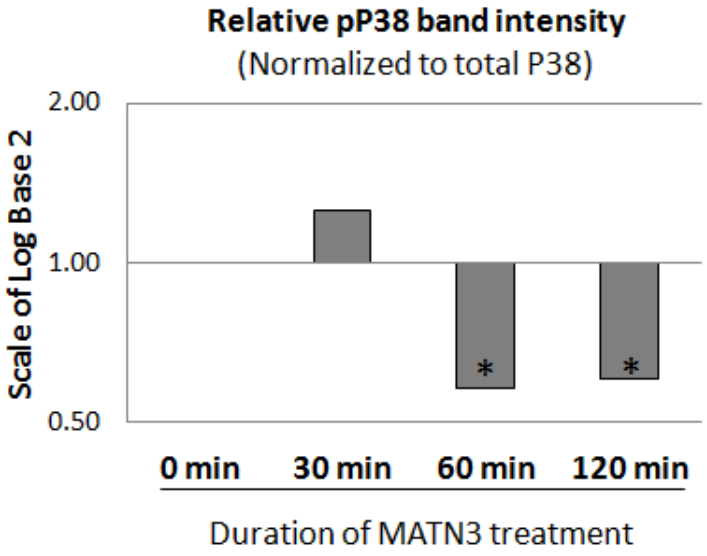

Relative pP38 band intensity
(Normalized to total P38)
Scale of Log Base 2
2.00
1.00
0.50
*
*
0 min
30 min
60 min
120 min
Duration of MATN3 treatment

Figure 4. MATN3 inhibits p38 phosphorylation and activation. Western blot analysis of phospho-p38 and total p38 band intensities in primary human chondrocytes treated with recombinant human MATN3 protein in a 0 to 120 minute time course **(A)**. Phospho-protein band intensities have been quantified and normalized to total protein band intensities using ImageJ software program **(B)**. MATN3 protein was used at a concentration of 200 ng/ml. Asterisks ($p \leq 0.05$) indicate statistically significant differences from the untreated control group. Data are representative of 3 individual experiments.

FIGURE 5

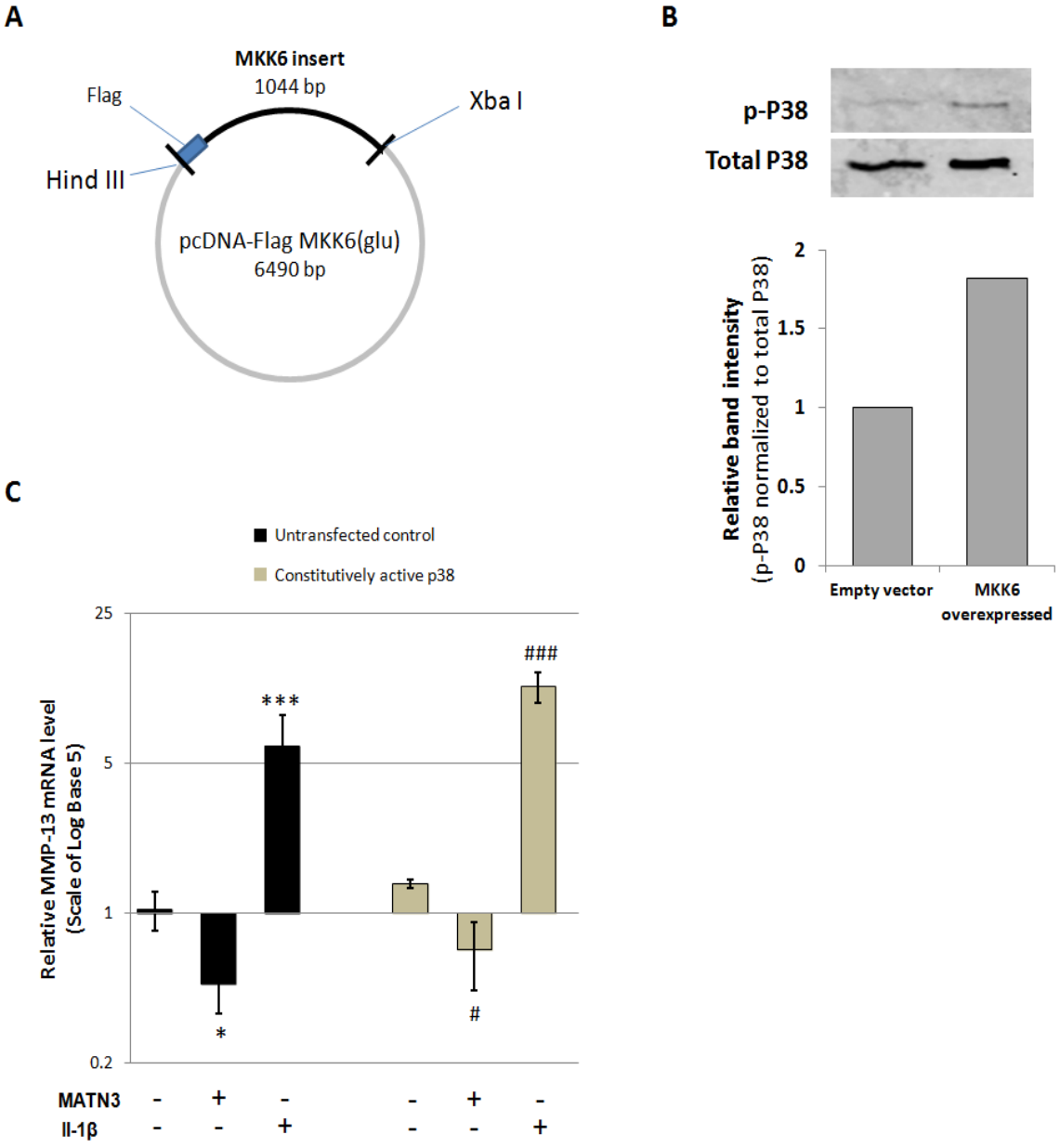

A
MKK6 insert
1044 bp
Flag
Xba I
Hind III
pcDNA-Flag MKK6(glu)
6490 bp
B
p-P38
Total P38
Relative band intensity
(p-P38 normalized to total P38)
2
1.5
1
0.5
0
Empty vector
MKK6
overexpressed
C
Untransfected control
Constitutively active p38
Relative MMP-13 mRNA level
(Scale of Log Base 5)
25
5
1
0.2
***
###
*
#
MATN3 - + - - + -
Il-1β - - + - - +

Figure 5. Constitutive activation of p38 does not affect MATN3 inhibition of MMP-13. Plasmid map of pcDNA3-Flag MKK6 (glu) vector construct, which carries the MKK6 transgene **(A)**. Western blot analysis of phospho-p38 and total p38 in primary human chondrocytes transfected with pcDNA3-Flag MKK6 (glu) or an empty pcDNA3 vector. Phospho-protein band intensities have been quantified and normalized to total protein band intensities using ImageJ software program **(B)**. Relative MMP-13 mRNA levels in untransfected chondrocytes vs. those that have been transfected with pcDNA3-Flag MKK6 (glu) and exhibit constitutively active p38 **(C)**. MATN3 protein was used at a concentration of 200 ng/ml and IL-1β was used at 5.0 ng/ml. Single asterisks ($p \leq 0.05$) and triple asterisks ($p \leq 0.005$) indicate statistically significant differences from the untreated and untransfected control group. Single hash marks ($p \leq 0.05$) and triple hash marks ($p \leq 0.005$) indicate statistically significant differences from the untreated but pcDNA3-Flag MKK6 (glu) transfected control group.

FIGURE 6

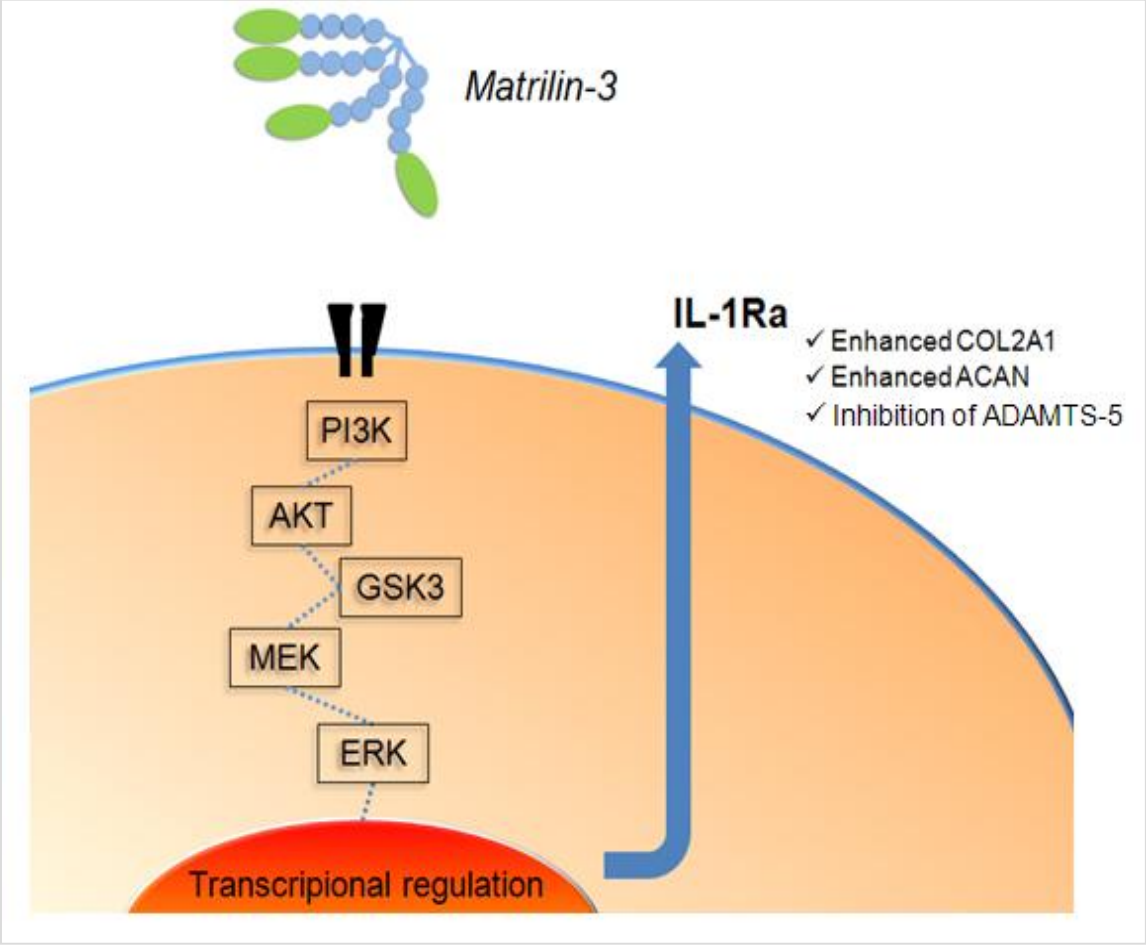

Matrilin-3
IL-1Ra
✓ Enhanced COL2A1
✓ Enhanced ACAN
✓ Inhibition of ADAMTS-5
PI3K
AKT
GSK3
MEK
ERK
Transcripional regulation

Figure 6. Proposed pathway: MATN3 regulates cartilage homeostasis markers through activation of PI3 kinase signaling. This diagram illustrates a possible biological mechanism that can explain MATN3's IL-1Ra dependent regulation of cartilage homeostasis markers type II collagen, aggrecan and ADAMTS-5 in chondrocytes. MATN3 is able to activate a chondrocyte cell receptor that leads to phosphorylation of PI3 kinase, Akt, GSK3, Mek and ERK1/2 ultimately stimulating IL-1Ra, which enhances type II collagen and aggrecan and inhibits ADAMTS-5. Because evidence presented in this study shows that EGFR activation is necessary to maintain several markers downstream of MATN3, this suggests that the unknown receptor may indeed be EGFR.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

Summary

In this body of work, we have investigated several novel characteristics of cartilage extracellular matrix (ECM) protein matrilin-3 (MATN3) that may protect against the onset and progression of osteoarthritis (OA). We have made several discoveries about the functional significance of MATN3 in cartilage tissue including its ability to promote chondroprogenitor cell differentiation and regulate homeostasis markers in chondrocytes. Moreover, we have uncovered much of the underlying biological mechanism by which MATN3 regulates such markers in chondrocytes.

Matrilin-3 inhibits OA conducive downstream effects of IL-1

Stimulation of cartilage anabolism

The questions that became the driving force of the present study were in many ways engendered by a remarkable finding made by our laboratory in 2006 that mice carrying a functionally deficient MATN3 gene, with a complete deletion of exon 2, were more susceptible to OA [1]. With the exception of having higher bone mineral density than their wild-type littermates, these MATN3 functional knock out (KO) mice developed normally. However, histological analysis of their hind limbs conducted 52 weeks after birth showed that MATN3 KO mice were afflicted by the presence of OA-like lesions in their joint cartilage indicating that the wild-type MATN3 gene may have the potential to protect cartilage from OA. To better understand the physiological cause of early OA onset in these MATN3 KO mice, we decided to measure the expression of the two main building blocks

of cartilage tissue: type II collagen and aggrecan. We observed that MATN3 KO mice exhibited a 50% reduction in the expression of both of these key cartilage components [2] suggesting that functional MATN3 can maintain the expression of these markers. Our *in vitro* studies seemed to corroborate these *in vivo* findings since we observed that- treating chondrocytes with recombinant MATN3 protein was sufficient to not only stimulate the expression of type II collagen and aggrecan, but also reversed the extent of IL-1 induced downregulation of these markers. The effect of MATN3 on IL-1 mediated inhibition of cartilage anabolic markers in chondrocytes is depicted in Figure 1.

Inhibiting the expression of aggressive proteolytic enzymes

It is well known that IL-1 can stimulate catabolic markers associated with cartilage degradation just as well as it can stimulate the downregulation of the aforementioned cartilage anabolic markers. Many of the most aggressive proteolytic enzymes produced by chondrocytes including collagenases (MMP-1, -3, -9, and -13) as well as aggrecanases (ADAMTS-4, -5) are upregulated during OA, and IL-1 can elevate their production in cartilage [3-10]. We discovered that treating chondrocytes with recombinant MATN3 protein resulted in an acute inhibition of IL-1 mediated induction of catabolic proteases MMP-13, ADAMTS-4 and ADAMTS-5. The effect of MATN3 on IL-1 induction of cartilage catabolic markers in chondrocytes is also depicted in Figure 1.

How can MATN3 influence IL-1 pathway targets?

Stimulation of a potent anti-inflammatory cytokine

In trying to understand how MATN3 can possibly hinder multiple tissue destructive effects of IL-1, we investigated whether MATN3 can stimulate any endogenous antagonists of the IL-1 pathway. Remarkably, we discovered that relatively low concentrations of recombinant MATN3 protein was capable of stimulating a significant increase of the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra), which is a IL-1 ligand mimic that binds IL-1 receptor with a much higher affinity than either IL-1α or β [11]. MATN3 treatment enhanced IL-1Ra expression in the presence and absence of IL-1β. We also tested the possibility that MATN3 could regulate other molecules such as IL-1 receptor I or IL-1 itself, which could potentially explain how MATN3 affects events downstream of IL-1. However, in both cases we found that MATN3 was incapable of regulating such events (data not shown in dissertation).

IL-1 receptor antagonist dependent and independent events

The first aim of this study focused on understanding which of the downstream effects of MATN3 were dependent and which were independent of IL-1Ra. By attenuating IL-1Ra mRNA and protein levels in chondrocytes using small interfering RNA (siRNA) based gene knockdown, we discovered that the induction of anabolic homeostasis markers, type II collagen and aggrecan, by MATN3 was severely hindered by this restriction of IL-1Ra. Furthermore, MATN3 seemed capable of inhibiting ADAMTS-5 expression only when IL-1Ra levels were not mitigated by siRNA. These findings clearly suggested that MATN3-mediated regulation of type II collagen, aggrecan and ADAMTS-5 in

chondrocytes were dependent on IL-1Ra. We found it rather intriguing that the regulation of MMP-13 by MATN3 was however completely unaffected by the attenuation of IL-1Ra in our *in vitro* system suggesting that MATN3-mediated regulation of this aggressive protease is independent of IL-1Ra. Perhaps even more intriguing was the observation that diminishing IL-1Ra levels via siRNA-based knockdown did not seem to significantly affect basal expression of MMP-13 in chondrocytes. Since IL-1 induction of MMP-13 is canonically regarded as a key driving force of OA associated tissue destruction, it is surprising that a potent inhibitor of IL-1 pathway signaling like IL-1Ra would have little to no effect on MMP-13 expression. In contrast, chondrocytes treated with IL-1Ra siRNA exhibited lower basal expression of type II collagen and aggrecan while their basal expression of the aggrecanase ADAMTS-5 was higher with respect to control cells, as expected. Taken together, these findings suggested to us that IL-1Ra regulates the physiological abundance of type II collagen, aggrecan and ADAMTS-5 (but not MMP-13) in chondrocytes. This is the reason why MATN3 can regulate these 3 homeostasis markers through the induction of IL-1Ra.

Potential application for OA therapy

Multiple animal studies have explored the benefits of exogenously introducing IL-1Ra (via protein injection or gene transfer) into articular cartilage tissue [12-15]. The attractive results of these studies, as well as reports by several clinical studies that found a negative correlation between OA severity and the abundance of IL-1Ra in diseased joints [16, 17], collectively contributed to much enthusiasm for the prospective use of IL-1Ra in OA therapy. Since the

possibility of using gene transfer technology in human patients is currently out of the question, one study investigated the clinical efficacy of remedying OA joints with intraarticular injections of IL-1Ra but ultimately fell short of the positive outcome that was so desperately desired [18]. It was later hypothesized that the relatively short half-life of soluble (s) IL-1Ra, which is approximately 4-6 hours in the serum, may account for failure of this trial. A clever way to circumvent the problem of injecting a therapeutic molecule that is as short-lived as IL-1Ra into the body as a remedy a chronic disease such as OA is to instead find ways to promote endogenous and long-lasting production of such molecules. The major appeal of utilizing an ECM molecule such as MATN3 to promote endogenous production of IL-1Ra by articular chondrocytes primarily lies in the longevity of its chondroprotective effect. The *in vitro* study conducted in Chapter 2 of this dissertation has yielded substantial evidence to suggest that MATN3 is capable of maintaining IL-1Ra stimulation for at least 36 hours following treatment. Moreover, the effects of MATN3 on homeostasis markers that are dependent on IL-1Ra, as well as those that are independent, persists for at least 24 hours post treatment. Furthermore, the utility of MATN3 as a possible future candidate for the purpose of OA therapy revolves around the principle of promoting the endogenous regulation of chondroprotective homeostatic events.

Matrilin-3 can regulate chondrogenic processes

Driving chondroprogenitor cell differentiation

Investigating how the absence or mutation of MATN3 is linked to the development of OA in animals and people has shed some light on its functional significance in the body. Evidence presented in Chapter 2 suggests that treating mature articular chondrocytes with recombinant MATN3 protein can prevent several harmful effects that are conducive to OA progression. In addition to its protective effects on articular chondrocytes, there is compelling evidence to favor the hypothesis that MATN3 can synergistically work with growth factors such as TGF-β to enhance the process of chondrogenic differentiation of cells [19]. With this in mind, it is important to recognize that in addition to chondrocytes, there also resides a progenitor cell population in articular cartilage tissue [20, 21]. These cells, termed chondroprogenitors, are mesenchymal stem cells that are destined for the chondrogenic lineage, but have yet to fully mature by chondrogenesis [22].

The focus of Aim 2 of this dissertation, as addressed in Chapter 3, was to investigate the effects of functional MATN3 on chondroprogenitors. In utilizing the ATDC5 murine chondroprogenitor cell line [23] to stably over express either the wild-type MATN3 (WT-MATN3) gene or one of 3 MATN3 mutations associated with HOA or chondrodysplasia, we were able to demonstrate that MATN3 promoted spontaneous chondrogenic differentiation of chondroprogenitors. Remarkably, overexpression of MATN3 or its mutant forms alone was sufficient to induce varying degrees of chondrogenesis in these cells as measured by Alcian blue staining and gene expression analysis. This finding strongly

supported the sentiment previously established by others who claim that some ECM proteins are capable of driving stem cell differentiation.

Chondrodysplasia associated mutations cause dramatic and permanent aberrations in the chondrogenesis of progenitor cells

The aberrations in chondrogenic potential between progenitor cells expressing WT-MATN3 and those expressing MATN3 mutants provided much insight into the functional significance of this molecule as well as the consequences of its mutation. When comparing the varying degree of chondrogenesis exhibited by these cells, Alcian blue staining and gene expression analysis via real-time PCR achieved similar conclusions. The MATN3 mutations corresponding to the most severe phenotypes in animals and patients appeared to have the highest negative impact on chondrogenic potential of these chondroprogenitor cells. As such, cells stably expressing the MATN3 mutation associated with spondyloepimetaphyseal dysplasia [24] (SEMD-MATN3) consistently exhibited the lowest Alcian blue staining intensities (indicating the lowest proteoglycan content), as well as lowest expression of aggrecan and type II collagen at all tested time points relative to all other transgenic progenitor lines. Likewise, cells expressing the MATN3 mutation linked to multiple epiphyseal dysplasia [25-28] (MED-MATN3), which subsequently leads to the second most severe phenotype in animals and patients, exhibited the second lowest aggrecan expression at all tested time points and the lowest type II collagen expression at most tested time points in all transgenic progenitor lines. These two chondrodysplasias-associated MATN3 mutations also caused substantial

elevation of the hypertrophic marker type X collagen. This finding suggested that in addition to dramatically hindering chondrogenic differentiation of progenitor cells, premature hypertrophy is an added consequence of disrupting the wild-type function of MATN3.

Hand osteoarthritis mutation and delayed signs of chondrogenesis

Although the hand osteoarthritis associated MATN3 mutant gene (HOA-MATN3) did not cause any significant changes to Alcian blue staining intensities relative to cells expressing WT-MATN3, it did however cause some alterations to aggrecan and type II collagen markers to suggest that it can delay chondrogenesic processes. The HOA-associated mutation greatly hindered the capacity of MATN3 to stimulate aggrecan and type II collagen expression in progenitor cells at the earlier time points tested. The induction of both of these key chondrocyte markers was suppressed by the HOA mutation as early as 24 hours after cells were seeded. While it was evident that aggrecan expression levels seemed to recover from this decline within 6 days of culture, the same was not true of type II collagen, whose expression remained suppressed at day 6 and only recovered by day 12. Upon further investigation, we discovered that the HOA-associated MATN3 mutation also suppressed cartilage homeostasis markers MMP-9 and MMP-13, which are normally elevated as immature progenitors differentiate into mature chondrocytes. This was observed also after 24 hours of culture and is therefore consistent with our aforementioned findings that the induction by MATN3 of chondrocyte marker expression is suppressed by the HOA-associated mutation, at early time points.

Matrilin-3 induction of IL-1 receptor antagonist – relevance to chondrogenesis

Strong stimulation of IL-1 receptor antagonist expression in chondroprogenitors

In Chapter 2 we demonstrated that MATN3 recombinant protein was capable of inducing the production of IL-1Ra in chondrocytes, which led to several downstream effects that could potentially prevent the progression of OA. Since patients with a thymidine to methionine (T→M) missense mutation in the first EGF-like domain of MATN3 are reported to be at a higher risk of developing HOA [29, 30], we decided to investigate whether this increased risk is due to potential differences between WT-MATN3 and the mutant HOA-MATN3 gene in their capacities to induce IL-1Ra, which has anti-OA properties [12, 15, 31-33]. In doing so, we quickly discovered that WT-MATN3 was indeed capable of inducing a significant elevation in IL-1Ra mRNA expression in chondroprogenitors. We observed that ATDC5 cells stably expressing WT-MATN3 exhibited an impressive 17-fold upregulation in IL-1Ra mRNA levels relative to parental controls. In contrast, ATDC5 cells expressing the HOA-MATN3 mutant gene was incapable of stimulating a significant increase in the mRNA expression of this anti-inflammatory molecule. Seeing how we had observed that the HOA-MATN3 mutation can delay the process of chondogenesis in progenitor cells, we next asked whether IL-1Ra stimulation is an integral prelude to the regulation of chondrogenic genes by MATN3.

Dependence of key chondrogenesis markers on IL-1 receptor antagonist

The second part of Aim 2 focused on exploring the possible connection between the acute enhancement of IL-1Ra expressionby MATN3 and its ability to promote spontaneous chondrogenesis of progenitor cells. We accomplished this goal by effectively dampening IL-1Ra from this biological equation to determine whether WT-MATN3 can carry on regulating chondrogenesis markers in its absence. Remarkably, genetically knocking down IL-1Ra prevented the stimulation of major chondrogenesis markers Col2a1, Acan and Sox9 by MATN3. These findings strongly suggested that spontaneous chondrogenesis of progenitors by MATN3 is IL-1Ra-dependent. In a way, this is not so surprising since several studies have previously shown that IL-1 can inhibit chondrogenesis of MSCs in a dose dependent manner [7, 34]. One of these studies went so far as to show that inhibiting IL-1 activation of NF-kB can rescue chondrogenesis in MSCs [7]. Given these reports, one would expect that a molecule such as IL-1Ra, which endogenously inhibits IL-1 pathway signaling, could therefore promote chondrogenesis. Overall, the induction of IL-1Ra by WT-MATN3 appears to play a key role in the regulation of chondrogenesic processes in ATDC5 murine chondroprogenitor cells. What is more, the defective regulation of IL-1Ra by HOA-MATN3 may help explain why cells transfected with this gene undergo delayed chondrogenesis.

Translatability: mouse to human model

In addition to helping us better place the functional significance of MATN3 in cartilage, our observation that MATN3 can induce the spontaneous

chondrogenesis of chondroprogenitors may also have potential clinical applications. Understanding the utility of chondroprogenitors for the purpose of regenerative medicine is currently a hot topic of investigation. Unlike mature articular chondrocytes, which quickly lose their chondrogenic phenotype upon *in vitro* expansion [35], chondroprogenitors can be expanded to a pre-hypertrophic state [36] and are therefore believed to be much more suitable for reintroduction into cartilage defects for the purpose of replenishing damaged tissue. However, before we can anticipate the clinical benefits of utilizing MATN3 as a driver of chondroprogenitor cell differentiation, it was important for us to first validate that the effects of MATN3 on murine chondroprogenitors accurately represents its function in their human equivalents. We did this by isolating primary human chondroprogenitors from the cartilage of Rhode Island Hospital (RIH) patients that underwent complete knee replacement surgery. Using gene expression analysis, we confirmed that the isolated cell populations were indeed chondroprogenitors through testing for MSC marker expression. As expected, these cells also exhibited lower expression of chondrocyte markers type II collagen and SOX9 compared to mature articular chondrocytes isolated from the same patients thereby verifying that they were an immature progenitor cell population. Treatment with MATN3 recombinant protein enhanced the expression of chondrogenesis markers aggrecan, type II collagen and SOX9 in these cells indicating that MATN3 can stimulate chondrogenic marker expression in human chondroprogenitor cells.

Is there a Matrilin-3 pathway?

Activation of PI3 kinase signaling

A question that was on our minds throughout the course of this entire study was: How can an ECM molecule, previously believed to have a purely structural purpose, behave like a growth factor in its regulation of homeostasis markers? To help address this question, Aim 3 of this dissertation focused on gaining a deeper understanding of the biological mechanism underlying the regulation by MATN3 of both IL-1Ra-dependent and independent downstream events. In this respect, we were fascinated to discover that within 30 minutes of treatment with MATN3 recombinant protein, chondrocytes exhibited rapid phosphorylation and activation of several signal transduction pathway molecules that are involved in PI3 kinase signaling including Akt, GSK3 and ERK1/2. This strongly indicated that MATN3 is capable of directly activating chondrocyte cell signaling. Moreover, previous studies had shown that the same kinases were involved in the induction of IL-1Ra by immune cells [37] suggesting that MATN3 can mediate direct regulation of this anti-inflammatory molecule through PI3 kinase pathway activation. To test this hypothesis we utilized the Ly294002 PI3 kinase chemical inhibitor to treat chondrocytes which were simultaneously exposed to MATN3 recombinant protein and observed that MATN3 could not mediate its IL-1Ra dependent regulation of type II collagen and aggrecan in the absence of PI3 kinase activity. We concluded that the effects of MATN3 o anabolic markers downstream of IL-1Ra are dependent on PI3 kinase pathway activity.

Epidermal growth factor receptor as a plausible mediator of MATN3 signaling

Since MATN3 was capable of initiating chondrocyte cell signaling relatively quickly, as observed in aforementioned experiments, we hypothesized that it may be interacting with a chondrocyte cell surface receptor to achieve this end. We had already established that PI3 kinase activity was essential for regulating several MATN3 downstream markers and so searched for chondrocyte cell surface receptors that were known initiators of PI3 kinase pathway signaling. The epidermal growth factor receptor (EGFR) stood out particularly well as a plausible MATN3 receptor not just because it can activate PI3 kinase signaling, but also because the protein structure of MATN3 contains several EGF-like domains that when mutated can lead to functional impairment of the entire molecule resulting in chondrodysplasia or arthropathy [24, 29]. *In vitro* chemical inhibition of EGFR activity in chondrocytes had adverse effects on the regulation of IL-1Ra by MATN3, as well as all cartilage homeostasis markers that we had previously shown to be downstream of IL-1Ra. In the presence of the EGFR inhibitor AG494, MATN3 was incapable of inducing a significant elevation in IL-1Ra, type II collagen or aggrecan mRNA levels. Similarly, AG494 hindered the capacity of MATN3 to inhibit of ADAMTS-5 mRNA expression. Taken together with the aforementioned PI3 kinase inhibitor studies, these findings supported the notion that both EGFR activity and PI3 kinase pathway activity are essential for mediating the effects of MATN3 on IL-1Ra-dependent downstream events.

Inhibition of 38 MAP kinase does not explain MMP-13 regulation

Our previous study showed that MATN3 is capable of inhibiting MMP-13 expression in chondrocytes in a manner that is independent of IL-1Ra [2]. In an effort to better understand the mechanism by which MATN3 regulates MMP-13 expression, we explored the possibility that MATN3 treatment leads to the inhibition of MAP kinase p38, which is known to be necessary for MMP-13 induction in chondrocytes [6]. Although it was observed that MATN3 treatment of chondrocytes led to an acute reduction in phosphorylated p38 levels, constitutive activation of p38 only mildly affected the capacity of MATN3 to inhibit MMP-13 mRNA expression. However surprising, this finding made it clear that the inhibition of p38 activation by MATN3 is not a critical step in the mechanism by which MATN3 regulates MMP-13.

Conclusions

This dissertation uniquely explores the functional significance of MATN3 with respect to cartilage tissue homeostasis and development. In the course of these studies, we make several novel discoveries highlighting the chondroprotective nature MATN3, including its stimulation of markers responsible for cartilage anabolism and its inhibition of OA-associated protease expression as well as its ability to initiate and drive chondrogenic differentiation of progenitor cells. We demonstrate that the induction of the anti-inflammatory cytokine IL-1Ra by MATN3 is at the heart of many of these chondroprotective functions and that EGFR-induced signaling and PI3 kinase activity are required to trigger such

downstream events. Overall, we have demonstrated that MATN3 is far more than a simple structural protein that bridges cartilage ECM networks, as was once believed. Its chondroprotective characteristics make MATN3 a molecule that could potentially have future use for promoting cartilage anabolism and healing in a clinical setting.

Future directions

A significant portion of our future studies will focus on positively identifying the remaining components of the MATN3 signal transduction pathway. Furthermore, we will continue to identify potential chondrocyte cell surface receptors that are candidate molecules through which MATN3 may signal. In this regard, we will try to further validate EGFR as a potential MATN3 sensitive receptor by analyzing the kinetics of its binding to soluble human MATN3 recombinant protein via solid phase binding assay or Biacore surface plasmon resonance (SPR). Secondly, we will focus on the *in vivo* efficacy of MATN3 as a deterrent of OA associated cartilage destruction. We will be working with Dr. Michael Ehrlich's group to identify the potential benefits of introducing recombinant MATN3 protein into a rabbit injury model of secondary OA.

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FIGURE 1

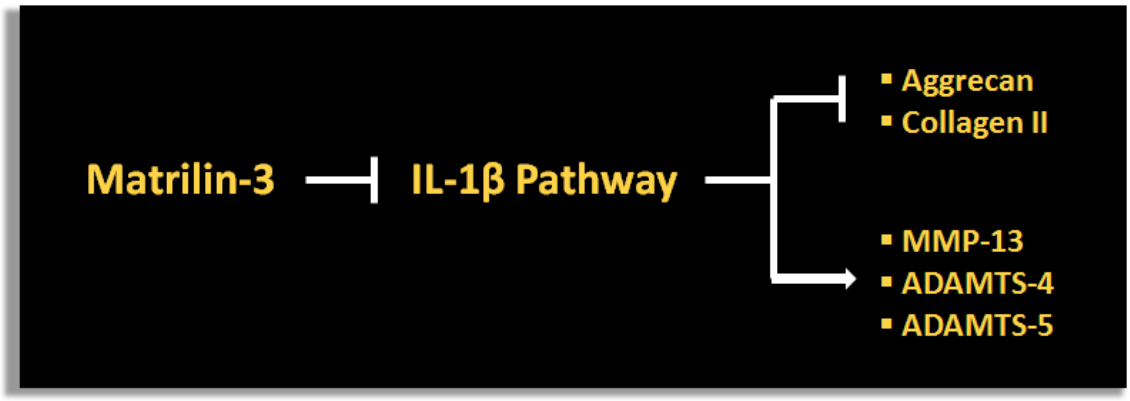

Figure 1. Matrilin-3 inhibits IL-1 pathway targets in chondrocytes. This illustration represents the ability of MATN3 to antagonize downstream effects of the IL-1 pathway. When uninterrupted, IL-1 pathway activity results in the inhibition of key markers of chondrocyte anabolism: aggrecan and type II collagen. It also stimulates the production of aggressive OA associated proteolytic enzymes such as MMPs and some members of the ADAMTS family of aggrecanases. MATN3 is capable of hindering these downstream effects mediated by IL-1.

FIGURE 2

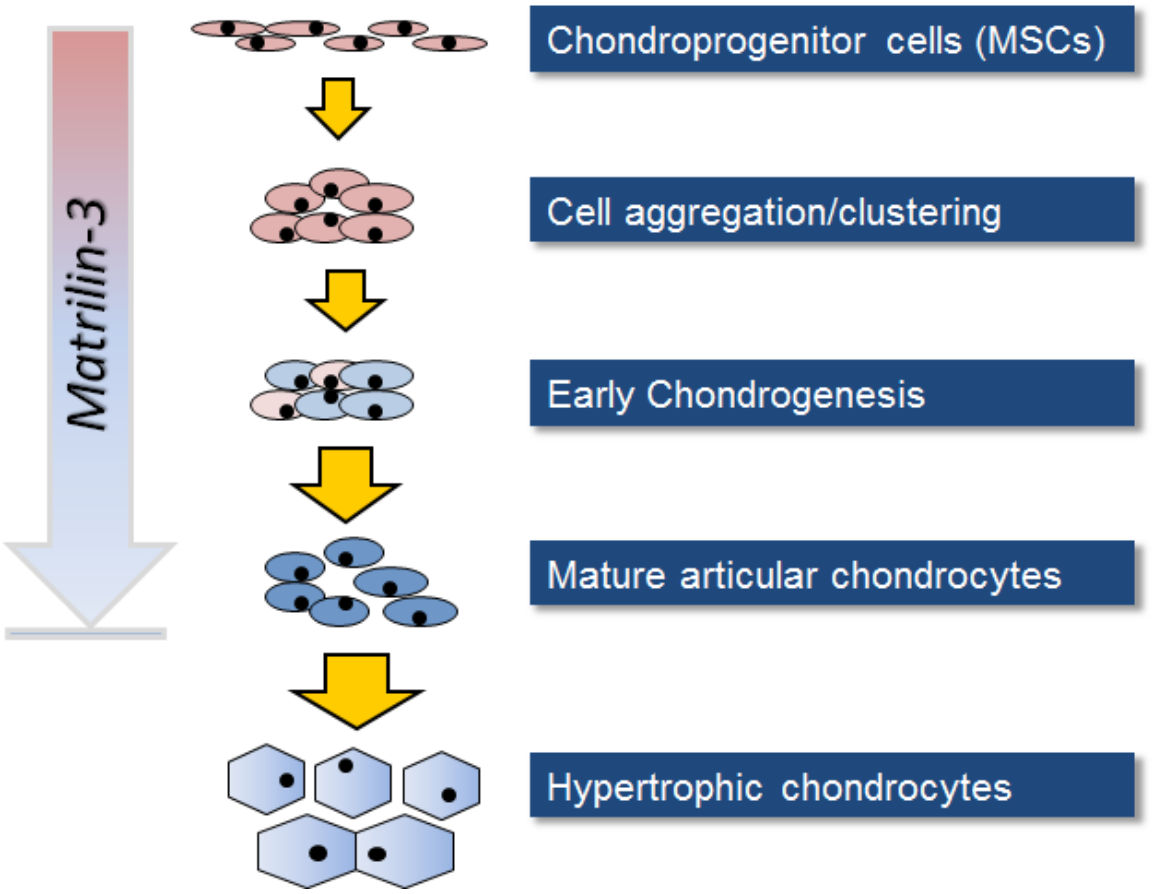

Figure 2. Matrilin-3 promotes progenitor cell differentiation into pre-hypertrophic chondrocytes. This diagram illustrates one of the functions of wild-type MATN3 in articular cartilage as it provides a microenvironment that is suitable for initiating and driving chondrogenesis of progenitor cells into pre-hypertrophic chondrocytes without promoting chondrocyte hypertrophy.

APPENDIX

TISSUE ENGINEERING: Part A
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Live-Cell, Temporal Gene Expression Analysis of Osteogenic Differentiation in Adipose-Derived Stem Cells

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Adipose-derived stem cells (ASCs) are a widely investigated type of mesenchymal stem cell with great potential for musculoskeletal regeneration. However, use of ASCs is complicated by their cellular heterogeneity, which exists at both the population and single-cell levels. This study demonstrates a live-cell assay to investigate gene expression in ASCs undergoing osteogenesis using fluorescently tagged DNA hybridization probes called molecular beacons. Three molecular beacons were designed to target mRNA sequences for alkaline phosphatase, type I collagen, and osteocalcin (*ALPL*, *COL1A1*, and *BGLAP*), genes characteristically expressed during osteogenesis. The percentage of cells expressing these genes in a population was monitored daily to quantify the uniformity of the differentiation process. Differentiating ASC populations were repeatedly measured in a nondestructive fashion over a 21-day period to obtain temporal gene expression data. Results showed consistent expression patterns for the investigated osteogenic genes in response to induction medium. Peak expression was observed at days 3–4 for *ALPL*, day 14 for *COL1A1*, and day 21 for *BGLAP*. Additionally, the differentiation response of sample populations became more uniform after 2 weeks in osteogenic induction medium, suggesting a syncing of ASCs occurs over time. These findings are consistent with previous studies of osteogenic differentiation and suggest that molecular beacons are a viable means to monitor the spatiotemporal gene expression of live, differentiating ASCs.

Introduction

Stem cell-based therapies hold immense promise for treating myriad diseases, and researchers across multiple fields have dedicated themselves to exploring this potential.[1] Tissue engineers frequently use stem cell populations induced with biochemical and/or biomechanical stimuli to generate tissues of interest, such as muscle or bone. Adipose-derived stem cells (ASCs) are especially attractive because of their relative abundance and nonimmunogenicity and have shown good potential for use in musculoskeletal regeneration.[2–4] However, experiments using ASCs are often confounded by heterogeneity, which can negatively affect cellular differentiation and matrix production. Single-cell and subpopulation effects are often obscured by the whole-population assays typically used by researchers.[5] A new method capable of nondestructively assessing stem cell differentiation and heterogeneity in populations over time would alleviate many of the issues currently faced by researchers in the field.

Mesenchymal stem cell heterogeneity exists at multiple levels. First, cell populations harvested from stromal tissues are nonuniform, containing a mixture of differentiated and undifferentiated cell types that can respond to environmental conditions in dramatically different fashions.[6] Second, the stem/progenitor cells themselves possess disparate differentiation capabilities (unipotency, bipotency, multipotency, and pluripotency).[7–9] This heterogeneity is problematic for both basic science experiments and translational applications because individual cells can only respond according to their capabilities. Understanding the degree of uniformity in differentiating populations is critical for identifying important subpopulations that hold the key to regenerating tissues and treating diseases.

This study establishes a live-cell analysis approach using fluorescently tagged DNA hybridization probes called molecular beacons to determine gene expression patterns in osteogenically differentiating ASCs. Molecular beacons are hairpin-shaped nucleic acid probes functionalized with a fluorophore and a quencher on opposing ends.[10] The loop

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Table 1. Molecular Beacon Sequences for Osteogenic Genes

Gene	*Beacon sequence 5′→3′*
GAPDH[28]	CGACGGAGTCCTTCCACGATACCACGTCG
ALPL	CGCTCCAGAGTGTCTTCCGAGGAGGTCAAGGAGCG
COL1A1	CGTCCCAAAAAAAAAAAAAAAAAGAAAAATATCAGGGACG
BGLAP	TCCGCCGGAAAGAAGGGTGCCTGGAGAGGAGCGGCGGA

Stem regions are underlined. Remainder of oligonucleotide forms the loop region, which is complementary to the gene of interest.

region of the probe is complimentary to a nucleic acid sequence of interest. In the absence of the target sequence, the probe retains its stem-loop structure and fluorescence is quenched. When the target sequence is bound by the loop region, the stem unfolds, affording fluorescence. Molecular beacons have been used in many capacities, including single-nucleotide polymorphism detection, real-time polymerase chain reaction (PCR) applications, and many live cell imaging applications.[11–16]

For this study, molecular beacons were designed to target mRNA molecules coding for alkaline phosphatase, type I collagen, and osteocalcin (*ALPL*, *COL1A1*, and *BGLAP*), genes characteristically expressed during osteogenesis.[17,18] By delivering novel beacons for genes expressed at progressive stages of osteogenic differentiation, we were able to investigate the uniformity of this process in living populations and identify spatiotemporal patterns of expression, which would not be possible using standard, destructive techniques.

The goal of this study was to analyze temporal gene expression patterns in living cells during osteogenic differentiation at the population and subpopulation levels. Human ASC populations were chemically induced along the osteogenic lineage over a 21-day period, and gene expression was quantified using custom-designed molecular beacons. This experimental approach marks the first time that an investigation of gene expression patterns in living mesenchymal stem cells has been performed in a repeatable, nondestructive fashion.

Materials and Methods

Cell culture

All cells were maintained in a humidified incubator at 37°C and 5% CO_2. MG-63 and HEK-293 cell lines (ATCC, Manassas, VA) were cultured in growth medium containing phenol red-free MEM (CellGro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 100 mM Glutamax, and 100 mM sodium pyruvate (ThermoFisher Scientific, Waltham, MA). Cells were passaged at 80% confluence using 0.25% trypsin-EDTA (ThermoFisher Scientific). For molecular beacon experiments, cells were seeded into 96-well plates at a density of ~25,000 cells/cm^2.

ASCs derived from subcutaneous adipose tissue, originally harvested from seven, healthy, nondiabetic donors between the ages of 18 and 60 years old, were purchased from Zen-Bio, Inc. (superlot #36; Research Triangle Park, NC). Cells were grown in expansion medium containing DMEM/F-12 (ThermoFisher Scientific), 10% FBS (Zen-Bio), 1% penicillin/streptomycin, 0.25 ng/mL transforming growth factor-β1, 5 ng/mL epidermal growth factor, and 1 ng/mL fibroblast growth factor (R&D Systems, Minneapolis, MN).[19] All ASCs used for experiments were at passage 4.

Beacon development and design

Three custom-designed beacons were developed corresponding to alkaline phosphatase, type I collagen, and osteocalcin mRNA (*ALPL*, *COL1A1*, and *BGLAP*, respectively), which are common markers of osteogenesis (Table 1). Each beacon was functionalized with a 6-FAM (excitation [Ex]: 492 nm/emission [Em]: 517 nm) fluorophore on the 3′ end and a Black Hole Quencher-1 on the 5′ end. A nucleic acid folding program, mfold, was used to model the secondary structures of each mRNA molecule based on thermodynamic stability.[20,21] The five structures with the lowest Gibbs' free energy were analyzed for regions of largely unpaired or looped secondary structure. A 20–30-base sequence was chosen and assessed using NCBI BLAST to ensure uniqueness.[22,23] ALPL, COL1A1, and BGLAP beacons were highly specific to their target sequences (e-values 10^5, 10^2, and 10^6 times smaller than the next sequence match, respectively). The stem region of each beacon was designed to give the probe an optimal melting temperature of 60°C–80°C.[24] The folding of the beacon sequence was also assessed to ensure that a hairpin structure existed. All beacons were manufactured and HPLC purified via commercial sources (MWG Operon, Huntsville, AL).

Molecular beacon hybridization assay

Validation of hybridization efficiency was done by measuring the fluorescence of fixed concentrations of beacon hybridization to varying concentrations of target sequence (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/tea). ALPL molecular beacon in pH 7.4 1× Tris-EDTA buffer (ThermoFisher Scientific; 100 μM solution) was added to wells in an opaque 96-well plate at a final beacon concentration of 5 μM/well. Stepwise concentrations of ALPL target sequence (0.5–5.0 μM) were then added to the wells. Controls included wells containing only beacon and Tris buffer, only target and Tris buffer, and only Tris buffer. Sample plates were incubated at 37°C for 10 min, and fluorescence was read with a spectrofluorometer (Spectramax Plus 384; Molecular Devices, Sunnyvale, CA; Ex: 492 nm, Em: 517 nm) every 10 min for a total of 270 min.[25]

Beacon validation and testing

MG-63 cells, which highly express osteogenic genes,[26] and HEK-293 cells were seeded at a density of 50,000–60,000 cells per well in a 24-well plate. Two nanograms of ALPL, COL1A1, and BGLAP molecular beacon (2 μL of 100 μM solution in Tris-EDTA buffer, pH 7.4) was each encapsulated in 4 μL xtremeGENE HP reagent (1:2 ratio beacon:reagent; Roche Biotech, Pleasanton, CA) and suspended in 100 μL

base medium (MEM) according to product instructions. The complexes were delivered to wells at a concentration of 0.5 μM to ensure that the molecular beacon would be in great excess of the mRNA transcripts ($\sim 6\times10^{12} - 10\times10^{12}$ beacons/well).[27] A previously published molecular beacon for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was encapsulated and delivered in the same way for use as a positive control.[28] Following beacon treatment, cells were allowed an uptake period of at least 2 h before being imaged on a Nikon Eclipse Ti-U epifluorescent microscope (Nikon Instruments, Inc., Melville, NY). Images were captured with a scope-mounted QICAM 12-bit digital camera (Qimaging, Surrey, BC, Canada). Signal intensity for presented figures was uniformly thresholded to minimize background levels.

Real-time quantitative polymerase chain reaction gene expression verification

mRNA was isolated from MG-63 cells and HEK-293 cells using the RNAqueous Kit (Ambion, Austin, TX) according to manufacturer's instructions. Real-time quantitative polymerase chain reaction (RT-qPCR) was conducted using the DNA Engine Opticon 2 (Bio-Rad, Hercules, CA) using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA). For RT-qPCR, 0.5 μg of RNA was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Species-specific primer pairs were used to compare *COL1A1* and *BGLAP* mRNA transcript levels in MG-63 and HEK-293 cells. Calculations were done using the delta delta Ct (ΔΔCt) method, normalized to rRNA 18S expression.

Osteogenic differentiation of ASCs

ASCs were chemically induced for osteogenesis following established protocols.[29] Control medium contained DMEM/F-12 (ThermoFisher Scientific), 10% FBS (Zen-Bio), and 1% antibiotic/antimycotic. Osteogenic induction medium included the addition of 1 nM dexamethasone, 21.6 mg/mL β-glycerophosphate, 50 μg/mL ascorbate-2-phosphate, and 10 μg/mL vitamin D3 (Sigma-Aldrich, St. Louis, MO). Two separate, 96-well plates were seeded with 30,000 cells/well in 24 wells per plate using control medium. All cells were treated with 1 ng/mL Hoechst dye to visualize nuclei (Sigma-Aldrich). After 24 h, 12 wells were given 180 μL osteogenic medium while the remaining 12 wells were given 180 μL control medium. Ninety percent of the medium was changed every other day for 21 days.

Beacon treatment and imaging of differentiating ASCs

mRNA-specific beacons were introduced to cells during the 21-day differentiation process. From days 2 to 10, four osteogenic wells and four control wells were treated with ALPL beacon as described previously and imaged daily. For days 8–16, four separate osteogenic and control wells were treated with COL1A1 beacon and imaged daily. For days 17–21, the remaining four osteogenic and control wells were treated with BGLAP beacon and imaged daily. Four fields of view in each well were taken of Hoechst-stained nuclei, fluorescent beacon signals, and bright field images of cells at 10× magnification (16 fields of view total for osteogenic/control conditions). Cells were treated with the appropriate beacons on days 2, 5, 7, 10, 14, and 17 to maintain saturating intracellular concentrations. This re-treatment schedule was chosen based on a beacon persistence assay in living cells that indicated that the signal was diminished by day 4 (Supplementary Fig. S2).

Image processing and analysis

CellProfiler image analysis software was used to generate a MATLAB-based algorithm that relates "child" fluorescent signals to "parent" Hoechst-stained nuclei.[30,31] This relation of fluorescence signal to parent nuclei is valid since no extracellular fluorescence was observed in any of the images. The software sets thresholding parameters for each image by first identifying the maximum and minimum values of pixel intensity in each image, then defining all pixel intensities in the lowest 20% as background. The program analyzed each set of images by first counting Hoescht-stained nuclei, which were recognized as ellipsoidal objects with a major axis between 5 and 20 microns (10 and 40 pixels). An area encompassing the nuclear/perinuclear region (25×10 μm ellipsoid) was defined when monitoring fluorescent signal in each cell. Individual fluorescent events were identified by pixel and grouped with the nearest nucleus, thus defining a cell as displaying positive signal or not (Fig. 1). The number of total fluorescing cells was divided by the total number of cells per image, giving a percentage of positively signaling cells for each sample well. Possible sources of error for this method included classifying weak signals as background, attributing signals to the incorrect cell due to overlapping nuclei, and discarding signals that were outside the analysis area. Despite these sources, however, the error rate from this method was only ± 8%, which is comparable to human error for similar samples. This analysis relied heavily on a preexisting modification to the CellProfiler program offered by the Broad Institute; the program and its modifications can be viewed at http://www.cellprofiler.org, and http://www.cellprofiler.org/CPmanual/RelateObjects.html.

Verification of osteogenesis

Alkaline phosphatase activity in differentiating ASCs was determined according to instructions for the BioVision alkaline phosphatase assay kit (Mountain View, CA). Briefly, four induced and four control wells per plate were either treated with ALPL molecular beacon or left untreated. After 7 days, these cells were lysed in 200 μL lysis buffer. Lysate was stored at −80°C until testing. For analysis, lysates were thawed and centrifuged at 13,000 rpm for 5 min. About 50 μL of the resulting solutions was transferred into individual wells of a 96-well plate and brought to volume with 110 μL of lysis buffer. The remaining 50 μL from each sample was transferred into separate wells on the same plate, brought to volume, and then treated with 20 μL stop solution to act as background controls. A standard curve using 0–0.5 mM alkaline phosphatase was made for quantification of samples. All wells were treated with 10 μL of 5 mM methylumbelliferone-4-phosphate solution for detection. The wells were covered and incubated at room temperature for 30 min, after which stop solution was added to all wells. A spectrofluorometer (Spectramax Plus 384; Molecular Devices) determined the fluorescence of each well at 360 nm/440 nm.

Alizarin Red-S (ARS; Sigma-Aldrich) staining was done for both control and osteogenically induced wells to examine

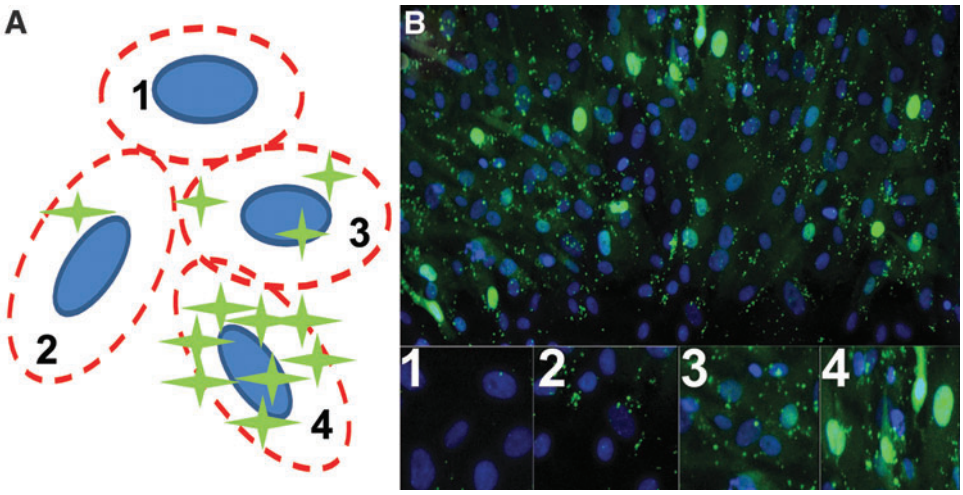

FIG. 1. The percentage of cells with positive signals for specific genes of interest was calculated using image analysis algorithms. A cartoon representation of the analysis is shown illustrating the basic concept **(A)**. Fluorescence signals were assigned to the nearest Hoescht-stained nuclei, establishing parent–child relationships between the two images **(B**, merged for illustration purposes**)**. "Low/no-signal" cells exhibited no fluorescence events in the region of interest above a minimum threshold value (A1, B1). "Positive" signals varied in type and intensity and included point signals (A2, B2), punctate, compressed speckling (A3, B3), and widespread fluorescence throughout the perinuclear region (A4, B4). All categories were included when calculating percentage of positive cells. Color images available online at www.liebertpub.com/tea

calcified matrix production. ARS (2% in distilled water) was pH-adjusted (4.1–4.3) and filtered through a 0.2-μm-pore filter prior to use. On day 21, beacon-treated wells were fixed with 3.7% paraformaldehyde in PBS (ThermoFisher Scientific). The fixed cell monolayers were washed in distilled water for 5 min, stained with 2% ARS for 20 min, and then thoroughly rinsed. After staining was complete, wells were imaged at 20× magnification using bright field microscopy and a scope-mounted digital camera (Labomed TCM 400; Labomed, Culver City, CA). ARS dye was then eluted with 10% cetylpyridinium chloride (ThermoFisher Scientific) overnight at 4°C, and the optical densities were measured at 540 nm with a spectrofluorometer (Spectramax Plus 384; Molecular Devices).

Statistical analysis

Gene expression patterns were determined using percent expression in differentiating ASC populations ($n=4$ for ALPL, COL1A1, and BGLAP). Data were analyzed using two-factor analysis of variance (treatment, time; $\alpha=0.05$) with Fisher's Least Significant Difference *post hoc* analysis. Osteogenic protein depositions were analyzed using a Student's *t*-test for control ($n=4$) and induced ($n=4$) samples.

Results

Molecular beacon hybridization assay

A 5 μM concentration of ALPL molecular beacon was used to assess hybridization to set concentrations of target sequence. At lower concentrations, the fluorescence intensity increased rapidly, while at the highest concentration the binding was saturated ($R^2=0.98$). Average fluorescence values increased threefold over a target concentration range from 0.5 to 5 μM (Supplementary Fig. S1). A signal-to-noise ratio of 24:1 was calculated based on these measurements, which is consistent with previous studies using molecular beacons.[32]

Beacon validation and testing

After administration of GAPDH beacon to MG-63 and HEK-293 cells, 97%–99% of cells in each sample population

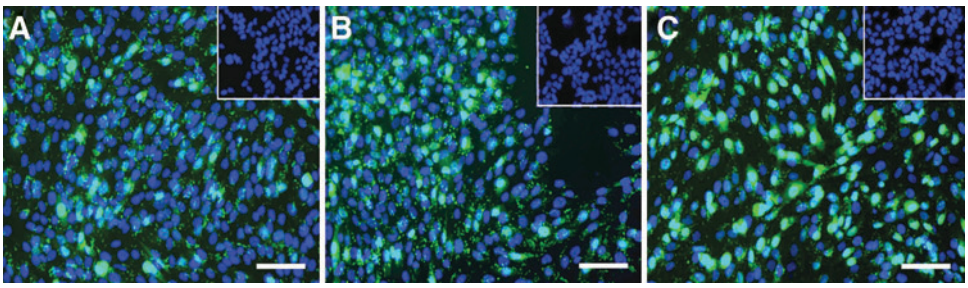

FIG. 2. Molecular beacons for *ALPL* **(A)**, *COL1A1* **(B)**, and *BGLAP* **(C)** were tested in MG-63 (positive control) and HEK-293 (negative control, insets) cells to ensure functionality and specificity. Fluorescence signals were observed throughout the MG-63 cells, while no signals were observed in HEK-293 cells. Color enhanced for presentation purposes. Scale bars: 100 μm. Color images available online at www.liebertpub.com/tea

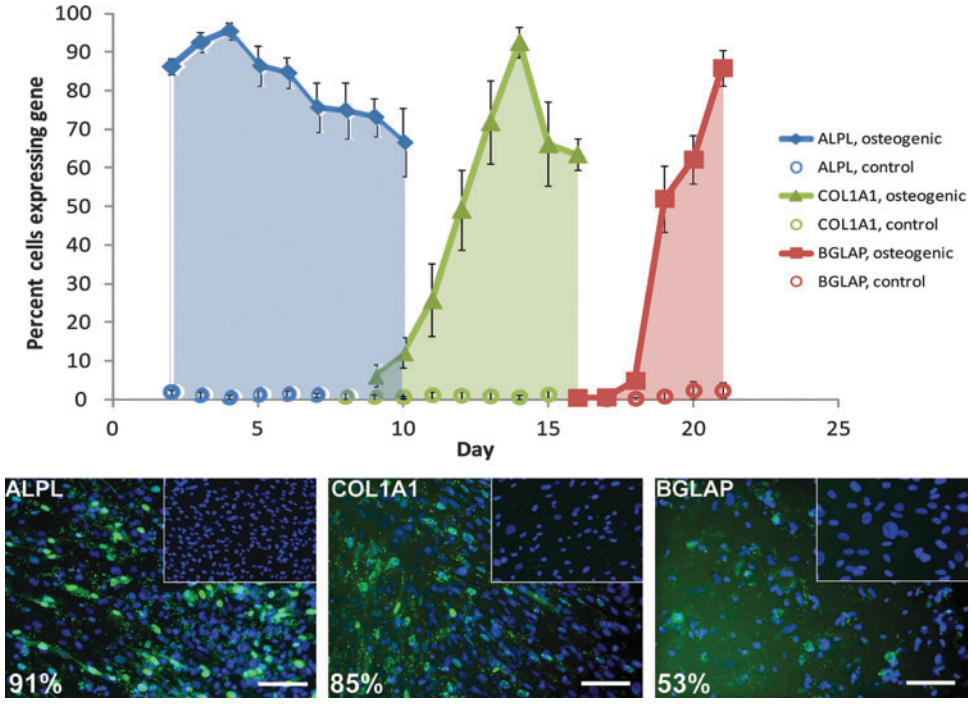

FIG. 3. Percentage of cells expressing *ALPL*, *COL1A1*, and *BGLAP* (induced, large insets; control, small insets. Scale bar: 50μm) measured daily from days 2 to 21. Expression percentages reflect the expected gene expression profiles for a differentiating population of adipose-derived stem cells (ASCs) (filled symbols). Peak percentages represent the point at which most cells in the induced population displayed positive signal for the gene of interest. These values can also be used as a measure of stem cell purity since only positively differentiating cells should express all three osteogenic genes. Control populations (open symbols) have signal levels close to zero, indicating a lack of osteogenic gene expression. Color images available online at www.liebertpub.com/tea

displayed positive signal. MG-63 and HEK-293 cells were treated with molecular beacons corresponding to *ALPL*, *COL1A1*, and *BGLAP*. About 95%–99% of treated MG-63 cells showed robust positive signal for all three beacons (Fig. 2). In treated HEK-293 cells, only 1%–2% of cells showed positive signal. All cells displayed typical morphology and remained spread over a 72-h observation period. To validate the expression levels of osteogenic genes detected by molecular beacons, we performed RT-qPCR to quantify the mRNA levels of *COL1A1* and *BGLAP* in beacon-treated MG-63 and HEK-293 cells. Both mRNA levels were greatly increased in MG-63 cells versus HEK-293 cells.

Molecular beacon signaling in differentiating ASCs

ASCs undergoing osteogenic differentiation were treated with molecular beacons for *ALPL*, *COL1A1*, and *BGLAP* in a stepwise fashion over 21 days. Percentage of positively signaling cells were calculated to monitor temporal gene expression patterns (Fig. 3). ALPL beacon-treated ASCs showed consistent positive signals during the first week of differentiation, starting at 86% on day 2, peaking at 92%–95% on days 3 and 4 ($p < 0.0001$), and decreasing to 63% on day 10. COL1A1 beacon-treated ASCs showed 6% positively signaling cells at day 9, rising to a peak of 91% on day 14 ($p < 0.0001$), and dropping off to 64% by day 16. Less than 1% of the ASC population showed positive BGLAP beacon signal from days 16 to 18, but the percentage of cells increased to 52% by day 19 and reached a peak percentage of 86% on day 21 ($p < 0.0001$), the final day of testing. Control ASCs that were not induced for osteogenesis showed less than 2% fluorescence in all instances.

Verification of osteogenesis

On day 7 of osteogenesis, both beacon-treated and untreated induced ASC samples had ~300 units of alkaline phosphatase activity per cell, while all control ASCs had ~5–10 units of activity per cell (Fig. 4; $p < 0.0001$). On day 21, the optical density of Alizarin Red dye eluted from osteogenically induced cells was three times higher than in control samples (Fig. 5A; $p < 0.0001$). Imaging of stained cells revealed a bright crimson color in osteogenically induced samples, while control samples retained little of the dye (Fig. 5B, C).

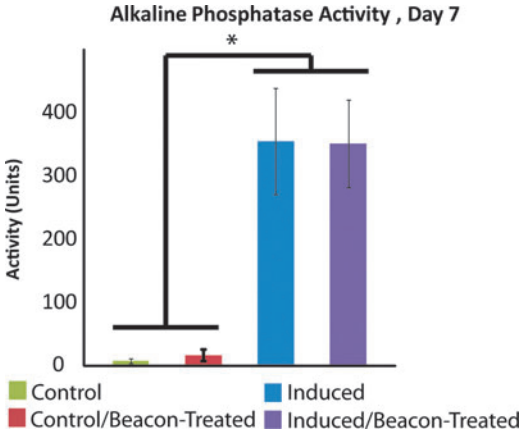

FIG. 4. Alkaline phosphatase activity in control and induced ASC populations was significantly different, regardless of beacon presence (*$p < 0.0001$). Activity in control samples was nearly zero, while activity in induced samples was two orders of magnitude higher. There were no significant differences in activity between beacon-treated and untreated samples undergoing osteogenesis, serving as both a signal of successful differentiation and evidence for uninterrupted protein synthesis in the presence of molecular beacons. Color images available online at www.liebertpub.com/tea

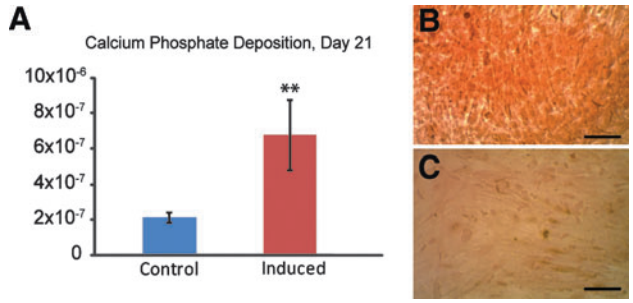

FIG. 5. Chemically induced ASCs successfully underwent osteogenesis over 21 days. Absorbance values for eluted Alizarin Red-S dye indicated that induced samples deposited three times more calcified matrix than control samples **(A,** $^{**}p < 0.0001$**)**. Induced **(B)** and control **(C)** ASCs stained with Alizarin Red-S for 30 min showed clear, qualitative differences in calcified matrix deposition. Scale bars: 100 μm. Color images available online at www.liebertpub.com/tea

Discussion

This study marks the first time that temporal gene expression patterns in living ASCs undergoing osteogenesis have been measured and quantified. Results suggest that the differentiation process in individual populations becomes more uniform over time. ASCs, like other mesenchymal stem cells, display considerable heterogeneity in their ability to differentiate in a uniform fashion, and few methods exist to investigate this behavior.[9] The goal of this study was to assess gene expression patterns during osteogenic differentiation at the subpopulation and population levels, thereby establishing a technique for investigating heterogeneity in differentiating ASC cultures. Differentiating ASC populations displayed clear, dynamic expression patterns of osteogenic genes, assessed by measuring the percentage of positively signaling cells over a 3-week period.

These experiments establish a technique to elucidate gene expression patterns and degree of uniformity during osteogenesis. By testing our system in established cell lines—MG-63 and HEK-293, and using a housekeeping gene, *GAPDH*—we were able to verify specificity and uptake of the beacon into >97% of the cells in a population. Subsequent measurement of the percentage of fluorescent, and therefore expressing, cells in the differentiating ASC populations showed a distinct pattern of upregulation followed by a slow decline in the number of expressing cells. Initial expression levels began lower for all genes (<10% for *COL1A1* and *BGLAP*) and then slowly rose to a peak of >90% in all cases. Thus, we were able to identify the peak expression times of characteristic genes in osteogenesis. An important point to note about this approach is that although the same samples were imaged each day, cellular proliferation and migration made it difficult to assess expression levels in the exact same cells over time. Hence, the "peak" in gene expression seen with our experiments reflects the time when the most cells have begun to express the gene of interest.

Interestingly, the percentage of positive signal cells increases in larger jumps during the later phases of osteogenesis. The ERK/Akt pathways have been implicated in osteogenic lineage commitment and have previously been induced by culture in type I collagen–coated flasks, implying that the presence of type I collagen in the extracellular environment may assist in committing a cell to the osteogenic lineage.[33] The data for *ALPL* gene expression show data points very close together in their slow rise and fall from the peak percentage point, with a difference of only a few percentage points between days 3 and 4. But as we map patterns for *COL1A1* and *BGLAP*, we see that the percentages jump from around 50% to 75% and from 60% to about 90%, respectively, over a single day. From these patterns, we could infer that the cells lock into osteogenesis after an initial induction period during which type I collagen is upregulated. While differentiation begins slowly in the first week, it quickly picks up speed and momentum, with more and more cells joining the differentiation process and settling into a more uniform gene expression pattern in later weeks.

Notably, each cell appears to be on its own track during differentiation. Some cells express the genes of interest very early on; for example, around 85% of cells express *ALPL* on day 2, though the peak percentage of cells expressing *ALPL* occurs on day 4. Cells that express genes early may also stop expressing genes sooner, which would explain the gradual decrease from the peak percentage as opposed to a steep drop. Additionally, when expression percentages are analyzed on a per-well basis, the changes in expression patterns from well to well are most apparent for *ALPL*, begin to even out for *COL1A1*, and are almost nonexistent for *BGLAP* (Supplementary Fig. S3). These expression patterns serve as additional evidence for the heterogeneity of stem cell populations, given the apparent differences in variation in gene expression from well to well during osteogenesis. Additionally, it is important to note the possibility that even when nearly every cell from a population is differentiating, not all of them so do in the same timeframe.

Use of molecular beacons can be complicated by a number of possible artifacts. One common concern is false signal due to nonspecific binding, probe instability, probe background noise, and probe degradation.[14,20,32–35] Nonspecific binding, or binding of probes to unintended targets, was not apparent in negative control HEK-293 cells. Likewise, noninduced ASCs treated with beacons showed almost no fluorescence, further indicating the absence of false-positive signals. We feel that these experiments are convincing evidence that nonspecific binding did not occur in our systems. Artifacts due to probe instability, background noise, and degradation were also not observed in the negative control cells. If the probes were unstable or degrading, a signal would be noticeable, either as a point source or by elevated background levels. Positive signal intensities were much higher in the positive control samples (MG-63 cells) than in any of the negative controls.[26] Independent of live-cell experiments, the beacon hybridization assay measured the background signal of probe alone, and data were normalized to this value, which was negligible compared with positive signals obtained in the same assay.

To confirm that osteogenic differentiation was successful, protein activity and deposition tests were performed on experimental samples. The alkaline phosphatase activity assay revealed protein activity two orders of magnitude higher in osteogenic samples than for controls, in both beacon-treated and untreated samples. There were no significant differences in activity between treated and untreated, induced samples, indicating that beacon hybridization to mRNA did not interfere with protein synthesis. Additionally, optical densities

of eluted AR dye showed three times higher levels in osteogenic samples compared with controls, and imaging of alizarin-red-stained cells revealed a scarlet coloring in osteogenic samples while control samples remained uncolored. Alizarin red staining for calcified matrix is a commonly used and well-established method for assessing osteogenic differentiation. Staining with alizarin red and testing for activity with the alkaline phosphatase assay were meant to ensure that differentiation had indeed occurred successfully, which would not have been possible had significant gene knockdown occurred.

While these methods are commonly used to assess success rates of osteogenic differentiation, they possess pertinent limitations that our molecular beacon-based analyses overcome. Both of these assays collect data from the differentiating population as a whole, meaning that whether an individual cell has successfully differentiated or not, it is lumped into the analysis. These nondifferentiated cells obscure contributions from differentiating cells and can yield unclear data. Additionally, quantification of the data is oversimplified by reporting each parameter on a per-cell basis; this again makes the incorrect assumption that all cells are contributing equally to the assay. The use of molecular beacons to quantify the rate of differentiation allows us to make accurate subpopulation- and population-based observations about a study by accounting for the heterogeneity present in the sample.

A practical limitation of this technique is its inability to quantify gene copy numbers within individual cells, akin to "gold standard," real-time PCR analyses. Theoretically, signal intensity from a beacon corresponding to an upregulated gene could be compared to the fluorescence intensity of a beacon for a housekeeping gene, such as 18S rRNA or *GAPDH*, but intensities of different fluorophores vary and are difficult to compare from cell to cell. Other limitations include the slight false-positive rate, indicated by the 1%–2% positive signal seen in HEK-293 cells, and the false-negative rate, indicated by the 1%–2% negative signal seen in MG-63 cells. These may be delivery method dependent, and thus could be solved for more sensitive assays. However, false-positive and -negative rates of 1%–2% were sufficiently small for the current study and had a minimal impact on the interpretation of the results.

A comparison between RT-qPCR and beacon data would provide interesting insight into the strength and weaknesses of both techniques. As such, we conducted validation tests on two cell lines with well-defined mRNA expression: HEK-293 and MG-63 cells. MG-63 (osteosarcoma) cells constitutively express our osteogenic genes, whereas HEK-293 (human embryonic kidney) cells do not. RT-qPCR data were collected for *COL1A1* and *BGLAP* to verify that high mRNA levels corresponded to extensive positive beacon signals in the same samples (Supplementary Fig. S4). As expected, MG-63 cells had much higher expression levels for osteogenic genes than HEK-293 cells, corroborating visual evidence of beacon signal differences. Additionally, many studies have been performed that assess the RT-qPCR–based gene expression patterns of osteogenically differentiating ASCs.[34–36] One of the strengths of RT-qPCR is that it allows for quantitative comparisons between groups, whereas molecular beacons only provide semi-quantitative comparisons associated with the number of cells expressing a given gene. In these validation experiments, we can quantify the difference in osteogenic gene copies using RT-qPCR between MG-63 and HEK-293 cells, but we cannot describe how that gene expression is distributed across the sample. Molecular beacons, while not providing hard mRNA copy numbers, can describe which portions of a population are expressing the genes of interest. Additionally, beacons can be used to repeatedly assess the same cell population over time. This is not possible for RT-qPCR, which requires lysing of the sample to obtain mRNA for reverse transcription. Well-to-well variations in RT-qPCR data could also be significant for heterogeneous samples, resulting in disparate differentiation behaviors for separate sample groups over time. To illustrate the value of having repeated measurement capabilities, we included a Supplementary figure showing the variation in the percentage of cells displaying positive signals for each mRNA target on a well-by-well basis (Supplementary Fig. S3). While not large, there are clear differences among the wells (e.g., *ALPL*, days 5 and 7). These differences lessen as differentiation proceeds (e.g., *BGLAP* shows uniform expression on almost all days). Molecular beacons provide opportunities to analyze data in this way, whereas conventional methods do not.

Increases in signal were attributed to upregulation of the genes of interest, while decreases were attributed to a cessation or downregulation of gene expression for the target sequence. However, other possibilities do exist. Beacon degradation was discussed previously as one but was shown to be only a possible contributor based on the low false-positive rates observed in HEK-293 cells. A second possibility involves cellular proliferation; as cells divide, the cytoplasmic concentration of beacons decreases, resulting in less fluorescence per cell. To counter this, cells were treated with beacon at multiple time points, not just initially, to ensure sufficient beacon concentration in all cells. Interestingly, other groups have reported the persistence of non-degraded, chemically modified molecular beacons up to 21 days after treatment, indicating their resiliency.[37]

The uniformity of differentiation apparent from the results indicates that the cell population used in this study contained very little heterogeneity. Freshly isolated ASCs have shown more heterogeneity and lower differentiation capability than a serially passaged population.[38] As a result, the peak percentages of cells expressing genes of interest during differentiation are very high, between 85%–95%. In freshly isolated, unpurified populations of ASCs, heterogeneity is expected to be more prevalent and could yield peak percentages lower than those seen here.

The current study revealed dynamic gene expression patterns for three characteristic genes in osteogenesis and established a method by which gene expression can be assessed in live cells over extended periods, findings that are useful for both continued studies of differentiation as well as elucidation of uniformity in differentiating populations. Molecular beacons can be developed for genes characteristic of other lineages and used to generate similar gene expression timelines. Additionally, stem cell populations from diverse sources could be monitored in this way to compare their heterogeneity and differentiation capability. Likewise, these beacons could also be used to determine the effectiveness of other differentiation media or stimulation techniques in live-cell, experimental designs.

Conclusions

Stem cell heterogeneity continues to be a research obstacle for both basic science experiments and clinical applications. Tissue-engineered constructs that rely on stem cells face this problem regularly, though it is largely undefined. This study proposes a novel approach to investigate gene expression in stem cell populations, which can be applied to assessments of uniformity in these populations. Live, osteogenically differentiating ASCs were assessed repeatedly over a 21-day period to obtain temporal gene expression data that help elucidate up- and downregulation of key, osteogenic genes in response to induction medium. Additionally, the percentage of actively differentiating cells in a population was quantified, providing a novel method to measure and define heterogeneity in stem cell populations. The demonstrated molecular beacon technology allows for acquisition of live-cell, gene expression data to clarify aspects of heterogeneity in these potentially transformative cell populations.

Author Contributions

E.M.D. and H.V.D. designed the study, analyzed all molecular beacon and protein deposition data, and wrote the article. H.V.D. conducted all live-cell molecular beacon and protein deposition experiments. I.S.V. conducted and analyzed the *in vitro* hybridization assay. Q.C. and C.J. designed and analyzed all RT-qPCR experiments.

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Disclosure Statement

No competing financial interests exist.

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