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Suppression of Sestrins in aging and osteoarthritic cartilage: dysfunction of an important stress defense mechanism

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SUMMARY

Objectives: Aging is an important osteoarthritis (OA) risk factor and compromised stress defense responses may mediate this risk. The Sestrins (Sesn) promote cell survival under stress conditions and regulate AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signaling. This study examined Sesn expression in normal and OA cartilage and functions of Sesn in chondrocytes.
Methods: Sesn expression in human and mouse normal and OA cartilage was analyzed by quantitative polymerase chain reaction (PCR) and immunohistochemistry. Sesn function was investigated by using small interfering RNA (siRNA) mediated Sesn knockdown and overexpression with analysis of cell survival, gene expression, autophagy, and AMPK and mTOR activation.
Results: Sesn mRNA levels were significantly reduced in human OA cartilage and immunohistochemistry of human and mouse OA cartilage also showed a corresponding reduction in protein levels. In cultured human chondrocytes Sesn1, 2 and 3 were expressed and increased by tunicamycin, an endoplasmic reticulum (ER) stress response inducer and 2-deoxyglucose (2DG), a metabolic stress inducer. Sesn1 and 2 were increased by tBHP, an oxidative stress inducer.
Sesn knockdown by siRNA reduced chondrocyte viability under basal culture conditions and in the presence of 2DG. Sesn overexpression enhanced LC3-II formation and autophagic flux, and this was related to changes in mTOR but not AMPK activation.
Conclusion: These findings are the first to show that Sesn expression is suppressed in OA affected cartilage. Sesn support chondrocyte survival under stress conditions and promote autophagy activation through modulating mTOR activity. Suppression of Sesn in OA cartilage contributes to deficiency in an important cellular homeostasis mechanism.

Introduction

Osteoarthritis (OA) is the most prevalent joint disease[1] with aging, obesity and joint injury as its major risk factors[1]. In established disease, all joint tissues are affected[2] while disease initiation appears to occur in cartilage and cartilage damage also appears to be a main driver of OA progression[3].

Pathogenesis mechanisms involved in cartilage degradation include chondrocyte death, the increased expression of extracellular matrix (ECM) degrading enzymes, inflammatory mediators, and reduced synthesis of new ECM components. There is also evidence for abnormal chondrocyte differentiation[4].

A recent concept that has emerged is a deficiency in important cellular defense mechanisms in aging and OA-affected cartilage contributes to the initiation and progression of tissue damage[4,5]. There is reduced expression of antioxidant enzymes, which in the setting of increased reactive oxygen and nitrogen species aggravates oxidative damage[6]. There is also a dysfunction in the removal of damaged organelles such as mitochondria and macromolecules due to defects in autophagy[5]. Intracellular signaling mechanisms that mediate these defective functions are reduced expression and activation of Forkhead-box class O (FoxO) transcription factors[7], AMP-activated protein kinase (AMPK)[8] and an apparent hyperactivation of mammalian target of rapamycin (mTOR)[9]. This is a central axis of cell signaling in OA that is linked to the Phosphatidylinositol-4,5-Bisphosphate 3-Kinase (PI3K) pathway and is involved in the cellular response to diverse types of stress[10].

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Sestrins (Sesn) were originally identified as downstream effectors of p53[11], and are involved in regulation of cell viability under different stress conditions. Sesn expression is induced by DNA damage in a pathway dependent on p53, and by oxidative stress, independent of p53. Sesn regulate cell function mainly through negative control of mTOR through AMPKα1 phosphorylation[12,13]. Sesn also can have direct control over autophagy by binding to p62 and regulating degradation of its substrate[14]. Sesn2 and p62 physically associate with Unc-51-like protein kinase 1 (ULK1), an autophagy-initiating protein kinase[15]. Sesn thus regulate autophagy through mTOR but also through different mTOR independent mechanisms.

This study is the first to analyze which Sesn are expressed in normal cartilage and to determine changes in aging and OA. We also examine the role of Sesn in the regulation of chondrocyte viability, mTOR activation and autophagy.

Methods

Cartilage donors

Normal human knee cartilage tissues obtained from tissue banks from three young normal female (age 35–46, mean 39.3 ± 5.8), six aging female (age 57–89, mean 71.8 ± 13.1) and 13 young normal male (age 18–49, mean 32.3 ± 10.4), 10 aging male (age 50–86, mean 66.1 ± 13.2) donors (approved by Scripps Institutional Review Board) and processed within 24–72 h post mortem. The cartilage samples from these young and aged donors were macroscopically normal. Full thickness cartilage was harvested for RNA isolation from identical locations on the medial femoral condyles. OA-affected cartilage was harvested from the tissue removed during knee replacement surgery from six female (age 42–75, mean 59.3 ± 11.3) and five male (age 60–71, mean 66.6 ± 4.34) donors.

Tissue processing and RNA isolation

Cartilage was stored at −20°C in Allprotect Tissue Reagent (Qiagen, Valencia, CA) immediately after resection from the subchondral bone. For RNA isolation, cartilage was pulverized in a 6770 Freezer/Mill Cryogenic Grinder (SPEX SamplePrep, Metuchen, NJ), and homogenized in Qiazol Lysis Reagent (Qiagen) using 25 mg tissue per 700 µl Qiazol. RNA was isolated using the miRNeasy Mini kit (Qiagen) with on-column DNAse digestion, followed by removal of proteoglycans using RNAmate (BioChain Institute, Newark, CA).

Quantitative polymerase chain reaction (qPCR)

RNA from cultured chondrocytes was isolated using Direct-zol RNA MiniPrep kits and TRI-Reagent (Zymo Research). Quantitative PCR was performed using a LightCycler 480 instrument (Roche Diagnostics). The following pre-designed TaqMan gene expression assays (Life Technologies) were used: Sesn1 (Hs00902787), Sesn2 (Hs00230241), Sesn3 (Hs00914870), Gapdh (Hs02758991_g1).

Immunohistochemistry

Immunohistochemistry was performed to assess protein expression patterns in human and mouse cartilage using the following rabbit antibodies: Sesn1 (Proteintech), Sesn2 (human: Abcam, mouse: Proteintech), Sesn3 (Proteintech). Rabbit IgG (1 µg/ml) was used as a negative control in all experiments. For human cartilage, expression patterns were compared between normal and OA samples. The methods for tissue processing and immunohistochemistry were described earlier[7].

Mouse knee joints

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at The Scripps Research Institute. In mice, we analyzed young normal and aged knees as a model of aging-related OA as described[16]. We also analyzed knees from animals with surgically induced OA[7].

In the spontaneous aging-related OA model, male and female C57BL/6J mice were kept under normal conditions and knee joints were collected at 6 and 18 months of age from at least 12 mice per time point. The surgical OA model was induced in 4 months old C57BL/6J mice by transection of the medial meniscotibial ligament and the medial collateral ligament (MMTL + MCL) as described[17] and animals were euthanized 10 weeks later. Knee joints from both murine models were resected from both hind legs, fixed in 10% zinc-buffered formalin for 2 days, and decalcified in TBD-2 for 24 h. Serial sections (4-µm-thick) were cut, and expression of Sesn proteins was analyzed by immunohistochemistry.

Chondrocyte culture

Chondrocytes were isolated from human knee cartilage as described previously[18]. In brief, cartilage slices were removed from the femoral condyles and washed in Dulbecco's minimal essential medium (DMEM). Tissues were then minced with a scalpel, transferred into a digestion buffer containing DMEM, 5% fetal bovine serum, L-glutamine, antibiotics, and 2 mg/ml clostridial collagenase (Sigma Chemical Co., St. Louis, MO) and incubated on a gyratory shaker at 37°C until the fragments were digested.

The isolated chondrocytes were plated at high density in DMEM with 10% CS and antibiotics and allowed to attach to the culture flasks. The cells were incubated at 37°C in a humidified gas mixture containing 5% of CO_2 balanced with air. The chondrocytes were used in the experiments at confluence (2–3 weeks in primary culture).

The immortalized human chondrocyte cell line T-C/28[19] was obtained from Dr. Mary Goldring. Cells were cultured in DMEM containing 10% CS and only cells that had been maintained for fewer than 20 passages were used in all experiments.

Cells were exposed to different stress conditions. We used 2-deoxy-glucose (2DG) as an inducer of metabolic stress, tunicamycin for endoplasmic reticulum (ER) stress and tert-Butyl hydroperoxide (tBHP) to model oxidative stress[18].

Plasmid and small interfering RNA (siRNA)

The siRNAs targeting Sesn1, Sesn2 or Sesn3 (s26032, s44570 and s38097, respectively; Life Technologies) or control siRNAs were transfected into chondrocytes using Lipofectamine RNAiMAX reagent (Invitrogen). All transfections were performed following the manufacturer's protocol. Experiments were carried out 24–48 h post-transfection, and cells were harvested 18–24 h thereafter for PCR and immunoblot analysis.

For overexpression, the Sesn2 (SC320354, OriGene) or mRFP-GFP-LC3 (ptfLC3, #21074, Addgene) or control plasmid were transfected into immortalized human T-C/28 chondrocytes[19] using Lipofectamine 3000 reagent (Invitrogen). All transfections were performed following the manufacturer's protocol. Experiments were carried out 24–72 h post-transfection, and cells were harvested for PCR and western blot analysis.