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

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Abstract

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 AMPK and 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 PCR and immunohistochemistry. Sesn function was investigated by using 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 stress response inducer and 2-deoxyglucose (2DG), a metabolic stress inducer. Sesn1 and 2 were increased by tBHP, an oxidative stress inducer.

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Author Contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Lotz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design: Shen, Alvarez-Garcia, Lotz

Acquisition of data: Shen, Alvarez-Garcia, Olmer

Analysis and interpretation of data: Shen, Alvarez-Garcia, Olmer, Li, Lotz

Conflict of Interest

The authors have no conflicts of interest.

Ethics approval

This study was conducted with the approval of the Human Subjects Committee and the Institutional Animal Care and Use Committee at The Scripps Research Institute.

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.

Keywords

Sestrins; osteoarthritis; mTOR; autophagy

Introduction

Osteoarthritis 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].

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 3 young normal female (age 35–46, mean 39.3±5.8), 6 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 hours 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 6 female (age 42–75, mean 59.3±11.3) and 5 male (age 60–71, mean 66.6±4.34) donors.

Tissue processing and RNA isolation

Cartilage was stored at −20ºC 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 25mg tissue per 700ul 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 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]. The surgical OA model was induced in 4 months old normal wild-type C57BL/6J mice by transection of the medial meniscotibial ligament and the medial collateral ligament (MMTL+MCL) and animals were euthanized 10 weeks later.

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, decalcified in TBD-2 for 24 hours. 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 hours post-transfection, and cells were harvested 18–24 hours 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 hours post-transfection, and cells were harvested for PCR and western blot analysis.

Immunofluorescence staining

Cells were fixed with 4% formaldehyde in PBS for 15 min. After washing three times with PBS, cells were counterstained with DAPI and then mounted in antifade agent on glass slides and visualized with an inverted fluorescence microscope (Olympus).

Cell viability assays

The MTT assay is a colorimetric assay for measuring cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes typically reflect the number of viable cells present. Following transfection with siRNA and incubation for 72 hours, cell viability was determined using MTT assay as described [18].

The CellTiter-Glo Luminescent Cell Viability Assay (Promega) is used to determine the number of viable cells based on quantitation of the ATP present. Following transfection with siRNA and incubation for 72 hours, cell viability was determined using the CellTiter-Glo Luminescent assay, according to the manufacturer's protocol. The results shown represent the mean ± 95% confidence interval of triplicate wells.

Analysis of autophagy

Autophagy activation was analyzed by the standard Western blotting for LC3 conversion [20] as described [21].

To detect the formation of autophagosomes and autolysosomes [22], cells were transfected with the mRFP-GFP-LC3 plasmid [23]. Immunofluorescence images of mRFP-GFP-LC3 were analyzed by standard protocols [23]. Because GFP is more sensitive than RFP to the acidic environment of lysosomes, the tandem RFP-GFP-LC3 protein will label autophagosomes as yellow (GFP/RFP) puncta, and autolysosomes as red (RFP) puncta [23]. Thus, quantification of yellow and red puncta allows the determination of autophagosomes and autolysosomes, respectively. The number of LC3-positive red or yellow puncta was counted in at least 20 cells per experimental condition.

Western blotting

At indicated time points, cultured human chondrocytes were lysed in RIPA buffer supplemented with Halt protease inhibitor cocktail and phosphatase inhibitor cocktail (Thermo Scientific) and samples were analyzed by western blotting as previously described [7]. The following antibodies were used: Sesn1, Sesn2, Sesn3 (Proteintech), pAMPK, AMPK, pS6, S6, LC3 (Cell Signaling), and Gapdh (Ambion).

Statistical analysis

For all data sets on human chondrocytes, at least three independent experiments with cells from different donors were performed. Each condition in each of the experiments was tested in duplicate (for PCR and western blotting) or triplicate (cell viability). When using the T-

C/28 cell line at least three separate experiments, each in duplicate or triplicate were performed.

Summary statistics for quantitative data are reported as means ± 95% confidence interval (CI), and for qualitative data as frequencies and percentages. Parametric t-tests or analyses of variance were used to assess differences between groups relating to quantitative variables, and chi-square tests were utilized with qualitative variables. *P*-values <0.05 were considered statistically significant. All analyses were done with SPSS statistical software, version 23.0 for Windows.

Results

Sesn expression in normal, aging and OA cartilage

The expression of Sesn mRNAs was analyzed by qPCR and this showed a significant reduction in Sesn1, Sesn2 and Sesn3 in human OA compared with normal cartilage (Figure 1A). Next, changes in Sesn protein expression in human cartilage were assessed by immunohistochemistry (Figure 1B,D). In young normal cartilage, approximately 50–70% of cells were positive for Sesn and the Sesn expressing cells were evenly distributed throughout all zones of cartilage. In old normal donors with no diagnosis or macroscopic evidence of OA, the percentage of Sesn2 and Sesn3 positive cells were significantly decreased in the superficial and medial zone compared with young normal cartilage. Sesn1, Sesn2 and Sesn3 positive cells in OA cartilage were also significantly decreased compared with normal cartilage.

In normal mouse knee joints, the percentages of positive cells were similar for Sesn1, Sesn2 and Sesn3 (Figure 1C,E). In C57Bl/6 mice with surgically induced OA, there was a significant reduction in Sesn1, Sesn2 and Sesn3 positive cells compared to normal knees (Figure 1C,E). In normal C57Bl/6 wild-type mice there was also a significant reduction in the expression of all three Sesn with aging (Figure 1C,E).

Regulation of Sesn expression in cultured chondrocytes

Normal human articular chondrocytes were exposed to different stress conditions to determine regulation of Sesn expression. Under basal conditions, low levels of expression were detected for all Sesn. Tunicamycin and 2-DG significantly increased mRNA levels of Sesn2 and Sesn3 at 6, 24 and 48 hours (Figure 2). Moreover, tunicamycin significantly increased RNA levels of Sesn1 at 48 hours. No changes in Sesn1, Sesn2 and Sesn3 RNA levels were detected in tBHP-treated samples at any time point. Concerning Sesn protein expression, tunicamycin and 2-DG significantly increased protein level of Sesn2 at 6, 24 and 48 hours. Moreover, tBHP increased protein level of Sesn2 and 3 at 6 hours. Western blot analysis showed similar to the mRNA levels, that the protein levels of Sesn changed in response to the stress stimuli (Figure 2).

Sesn and chondrocyte viability

Individual and combinations of Sesn siRNAs were transfected into chondrocytes to determine the role of Sesn in regulating cell viability. Transfection with siRNAs reduced

Sesn1 mRNA levels by 80%, Sesn2 mRNA levels by 60% and Sesn3 by 88% (Figure 3A). Protein levels were reduced by 50% for Sesn1, 60% for Sesn2 and by 65% for Sesn3 (Figure 3B). Sesn3 mRNA levels were consistently decreased by transfection with siRNAs targeting Sesn1 by 10% and Sesn2 by 30%. This effect was not detected at the protein level. Sesn1 or Sesn2 mRNA or protein levels were not affected by transfection of siRNAs targeting the other Sesn.

Under basal culture conditions, only the combination of siRNAs for the three Sesns but not the individual Sesn siRNAs significantly reduced cell viability (Figure 4A). In response to treatment with the stress inducers tunicamycin, 2DG and tBHP, there was a dose-dependent and significant reduction in cell viability and this was further reduced by siRNA for Sesn1, Sesn2 and Sesn3 (Figure 4B,C).

Sesns control of mTOR activity and autophagy

We analyzed mTOR signaling upon knocking down all Sesn or overexpressing Sesn2 in the human T-C/28 chondrocyte cell line [19] under basal conditions and upon treatment with Earle's balanced salt solution (EBSS) to induce starvation. We focused in Sesn2 as this Sesn showed the largest differences in expression following exposure to stress conditions (Figure 2) and Sesn 2 inhibits mTOR and might play a more important role in stress responses than other Sesn family members [13, 15, 24, 25]. As shown in Figure 5A,C, S6 phosphorylation levels were significantly increased in cells treated with siSesn2. Conversely, Sesn2 overexpression with or without EBSS treatment also decreased phosphorylation of S6 (Figure 5A and 5C). However, knockdown of all Sesn or overexpression of Sesn2 did not affect AMPK phosphorylation.

As mTOR is a negative regulator of autophagy [26], we assessed whether Sesn affect autophagy through controlling mTOR activity. Firstly, we monitored changes in the levels of the autophagy marker LC3 in T-C/28 cells after knockdown or overexpression of Sesn, followed by treatment with the autophagy inducers EBSS or rapamycin (Figure 6A–D). Treatment of cells with control siRNAs significantly increased LC3 as compared to the DMSO only control. This is likely due to effects of the transfection reagent lipofectamine [27]. Notably, overexpression of Sesn2 increased the relative amount of LC3-II under normal conditions or after EBSS or rapamycin treatment. However, knocking down Sesn2 or all Sesn did not affect LC3-II formation (Figure 6A–D).

To analyze autophagic flux, we also monitored the formation of autophagosomes and autolysosomes following transfection of mRFP-GFP-LC3 [23]. As shown in Figure 6E,F, overexpression of Sesn2 under basal conditions or under rapamycin treatment led to a 2-fold increase of LC3 puncta (both yellow and red), indicative of increased autophagic flux.

Discussion

This study is the first to report that normal articular cartilage expresses Sesn and that OA and aging are associated with reduced expression of Sesn. In cultured chondrocytes, Sesn are induced by various types of extracellular stress and regulate cell survival, mTOR signaling and autophagy activation.

Sesn were originally identified as p53 induced genes [11]. In certain cancer cells, Sesn2 can also be induced in a p53-independent and JNK-dependent manner [11, 28]. The major physiological functions of Sesn are in the regulation of metabolic homeostasis via control of mTOR activation [29] and their potential function of antioxidants [11]. Sesn1 and 2 reduce oxidative stress by rescuing the peroxidase activity of overoxidized peroxiredoxins [30] and by activating the Nrf2 (nuclear factor erythroid 2-related factor 2)-Keap1 (Kelch-like ECH-associated protein 1) pathway [14], which induces antioxidant genes. Our data show that Sesn expression in chondrocytes is induced by various stressors, which also can induce cell death. Knockdown of Sesn under treatment with these stressors significantly reduces cell viability in cultured chondrocytes, indicating that they convey essential cellular protective functions.

Sesn2 inhibits activation of mTOR [25], a prometabolic serine/threonine kinase that controls protein synthesis, cell growth, autophagy and cell death and conveys stress signals for the reprogramming of cellular metabolism and the restoration of organismal homeostasis [29]. Genetic deficiency of Sesn2 in mice is not associated with any gross developmental abnormalities [31] but exacerbates obesity-induced mTOR activation, glucose intolerance, insulin resistance and results in diverse age- and obesity-associated metabolic pathologies such as accumulation of lipid droplets and protein aggregates, mitochondrial dysfunction, and muscle degeneration [24, 29]. Sesn2-deficient mice fail to inactivate mammalian target of rapamycin complex 1 (mTORC1) in the liver during fasting [14], and spontaneously elevated mTORC1 signaling is observed in mice devoid of both Sesn2 and Sesn3 [29]. Mice deficient in all Sesn exhibit reduced postnatal survival associated with defective mTORC1 inactivation in multiple organs during fasting [32]. Persistent mTOR activation is associated with diverse diseases and metabolic disorders [33]. mTOR hyperactivation has been observed in OA cartilage [9] and mTOR inhibition with rapamycin [34] or cartilage specific deletion [35] reduced the severity of experimental OA in mice. Our present data show that Sesn overexpression or knockdown in chondrocytes modulates mTOR activation as indicated by changes in the phosphorylation of the mTOR target S6.

A potential mechanism for mTOR hyperactivation is misregulation of AMP-activated protein kinase (AMPK). AMPK is a central nutrient and energy-sensor that is activated under stress conditions such as hypoxia, glucose starvation [36]. AMPK regulates the activity of proteins associated with mammalian target of rapamycin complex 1 (mTORC1), including tuberous sclerosis protein complex 2 (TSC2) and raptor, thereby regulating the activity of mTORC1. Sesn2 associates with AMPK and promotes its activation by an upstream kinase LKB1 [13, 25]. This activation of AMPK can result in mTORC1 inhibition and subsequent autophagy activation [37]. Sesn2-deficient cells are unable to promote autophagy under certain stress conditions [37]. AMPK protein and phosphorylation levels are reduced in OA [8]. The abnormal Sesn expression and AMPK activation are two mechanisms that converge to lead to mTOR hyperactivation in OA. However, in the present study the modulation in mTOR activation following Sesn knockdown or overexpression was not accompanied by changes in AMPK phosphorylation, suggesting that, under the experimental conditions used, the effect of Sesn on mTOR is not dependent on AMPK in chondrocytes. Our findings are in concordance with recent reports showing that Sesn also inhibit mTORC1 activity in the absence of AMPK [32]. This AMPK-independent mechanism involves Sesn

binding to the heterodimeric RagA/B-RagC/D GTPases resulting in suppression of mTOR lysosomal localization [38].

The present results also show that Sesn knockdown are not only associated with reduced mTOR activation as measured by phosphorylation of S6 but also of with changes in autophagy. We observed differences in the levels of LC3II as well as differences in the levels of autophagosomes and autolysosomes following modulation of Sesn levels by overexpression of knockdown in chondrocytes. Defects in autophagy have previously been reported in OA-affected and aging cartilage [16, 39].

Although a role for Sesn in cellular aging has been postulated based on findings in model organisms with Sesn gene deletion [40], the present findings are to our knowledge the first to show an aging related reduction in the expression of Sesn. This was observed in articular cartilage during aging of human and mouse knee joints. Sesn expression was also reduced in the surgical knee destabilization model in mice. Since Sesn1 and Sesn3 are target genes of FoxO transcription factors [24, 41], the reduced Sesn expression in aging and OA cartilage could be in part due to a reduction in FoxO expression [7].

The present study has the following limitations. The results on the role of Sesn in regulating cell survival under stress conditions are obtained with primary chondrocytes where Sesn were knocked down with siRNA. In our hands, the transfection efficiency in human chondrocytes is too low to achieve sufficient overexpression of proteins using plasmids and regulation of cell survival in chondrocyte cell lines is very different from primary chondrocytes in which we performed these key experiments.

In conclusion, Sesn mRNA and protein levels are reduced in OA-affected and aging human and mouse cartilage. Sesn support chondrocyte survival under stress conditions, promote autophagy activation and modulate mTOR activity. Suppression of Sesn in OA cartilage contributes to deficiency in an important cellular homeostasis mechanism.

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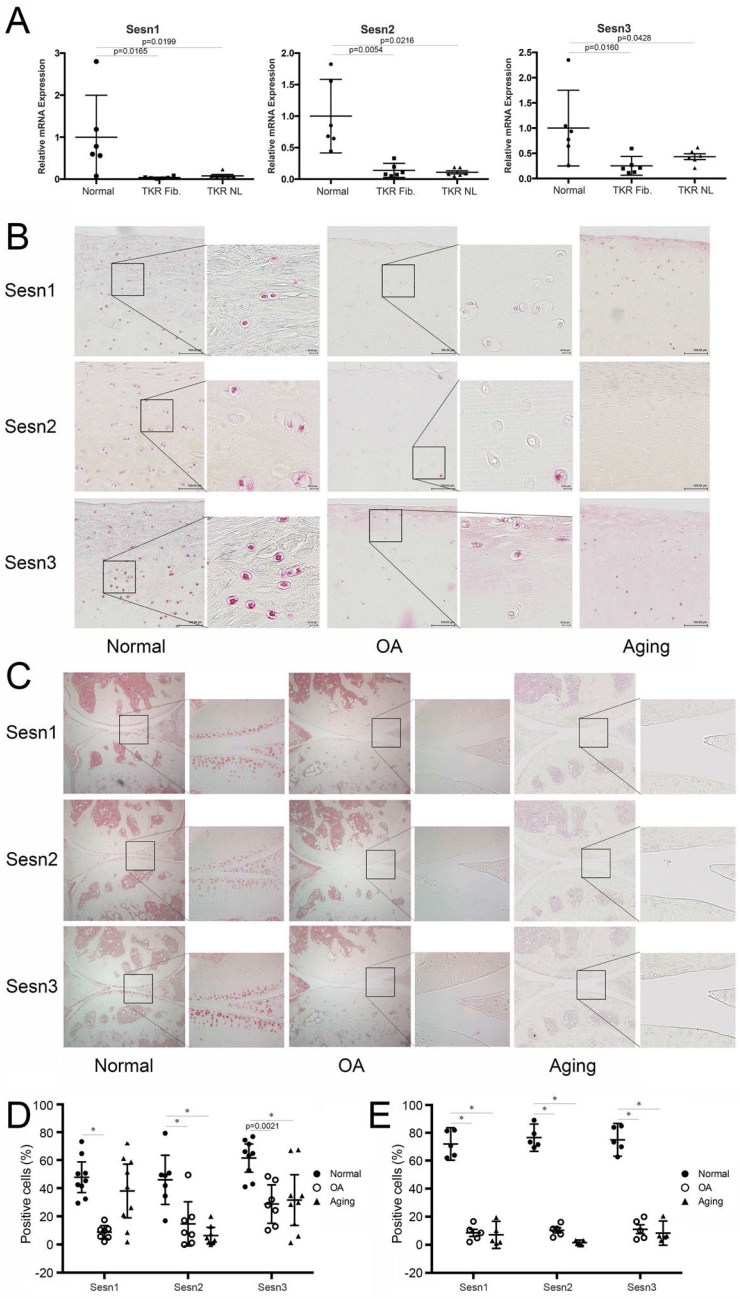

Figure 1. Sesn mRNA and protein expression in articular cartilage

A) Sesn1, Sesn 2, and Sesn 3 mRNA levels were analyzed by quantitative PCR in cartilage samples from normal (N=6) and OA (N=6) donors. TKR Fib: total knee replacement, fibrillated area. TKR NL: total knee replacement, normal appearing area. Data are expressed as mean ± 95% confidence interval (CI). Data were analyzed by paired-samples t-test. * = $P < 0.05$ versus normal group.

B) Immunohistochemical analysis of Sesn expression in human cartilage collected from normal (N=9), aging (N=9) and OA (N=7) knee joints. Representative images are shown. Images are 10× and insets on the right are 40× magnification.

C) Immunohistochemical analysis of Sesn protein expression in mouse joints from normal (6 months old, N=5), aging (18 months old, N=5) and DMM induced OA (N=5). Representative images are shown. Images are 10×and insets are 40× magnification.
D) Quantification of Sesn1, Sesn2 and Sesn3 immunopositive cells in human cartilage superficial and mid zones. Data are expressed as % positive cells. Dara were analyzed by chi-square test. * = $P < 0.05$ versus normal group.
E) Quantification of Sesn1, Sesn2 and Sesn3 immunopositive cells in mouse articular cartilage. Data are expressed as % positive cells. Data were analyzed by chi-square test. * = $P < 0.05$ versus normal group.

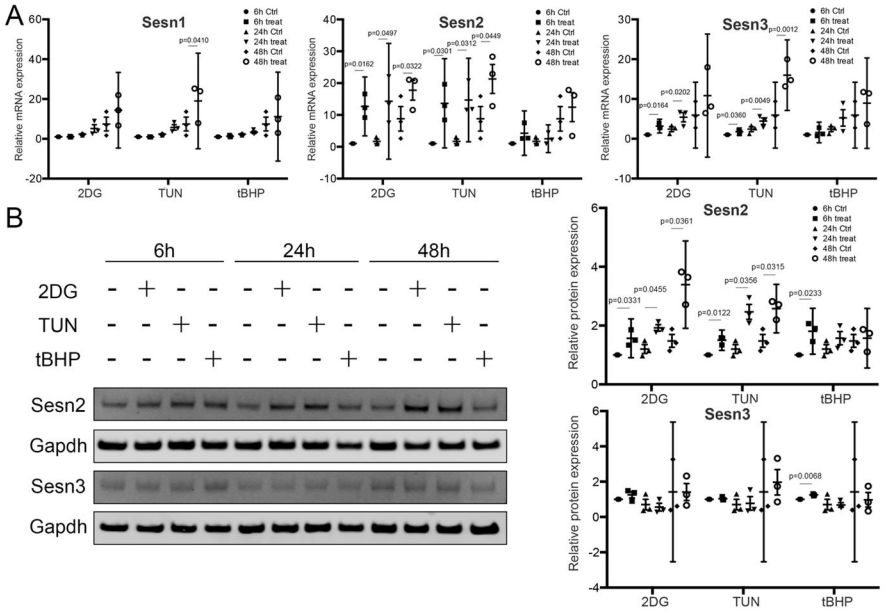

Figure 2. Changes in Sesn expression in response to stress inducers in cultured human chondrocytes

A) mRNA levels for Sesn1, Sesn2 and Sesn3 assessed by quantitative PCR in human articular chondrocytes treated with 2-deoxy-D-glucose (2-DG; 20mM), tunicamycin (1ug/ml), or tBHP (250uM) for 6, 24 and 48 hours. Data are expressed as mean ± 95% CI. Data were analyzed by paired-samples t-test. * = $P < 0.05$. Graphs represent quantitative PCR data from three different experiments using cells from different donors, each performed in duplicate.

B) Protein levels for Sesn2 and Sesn3 assessed by Western blotting of human articular chondrocytes treated with the indicated stress inducers for 6, 24 and 48 hours. Representative western blot images are shown. Graphs represent quantification of three different experiments each performed in duplicate. Data are expressed as mean ± 95% CI. Data were analyzed by paired-samples t-test * = $P < 0.05$.

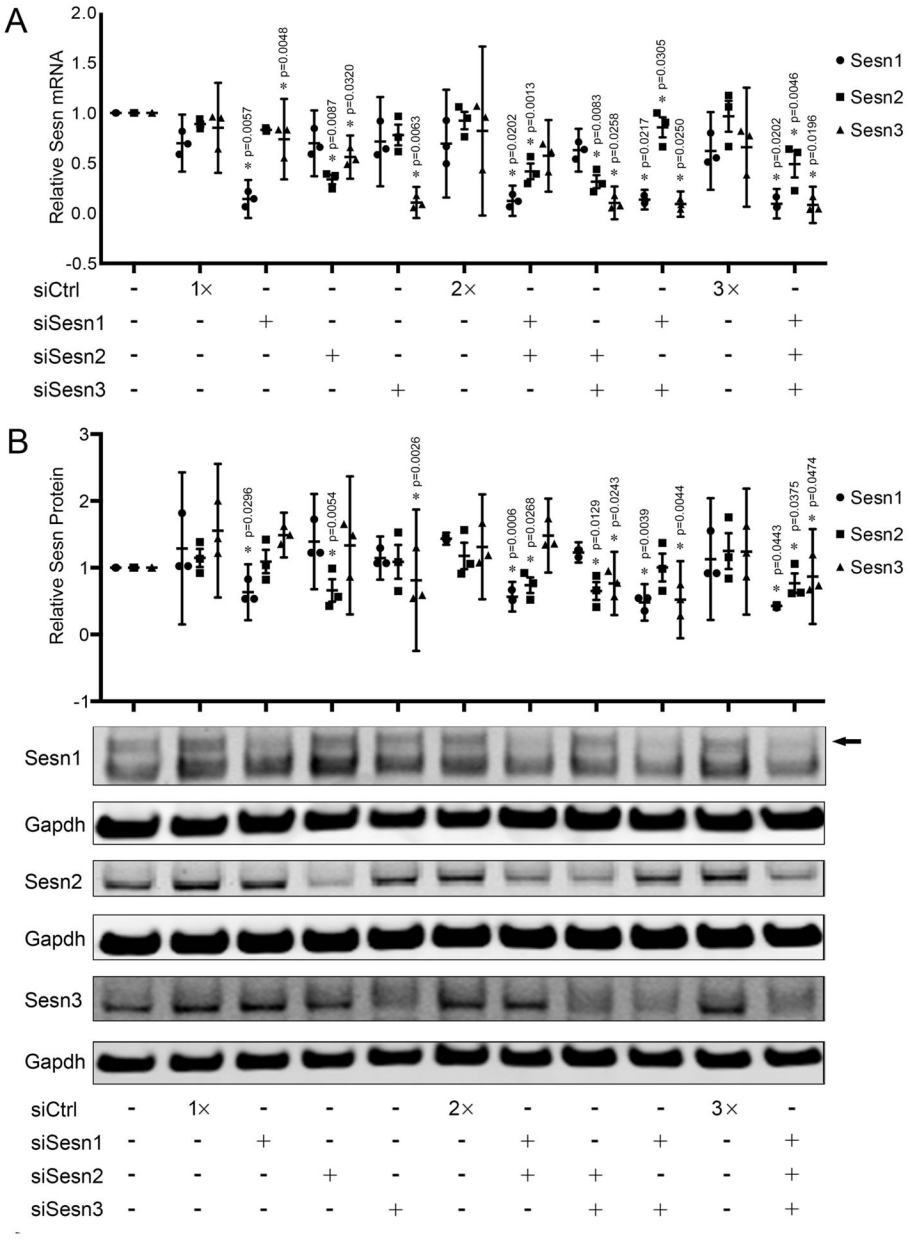

Figure 3. Specificity of Sesn siRNAs

A) Sesn1, Sesn2 and Sesn3 mRNA levels analyzed by quantitative PCR in human articular chondrocytes transfected with small interfering RNA (siRNA) targeting Sesn (siSesn). Data are expressed as mean ±95% CI. Data were analyzed by paired-samples t-test. * = $P < 0.05$ versus siCtrl. Graphs represent quantitative PCR data from three different experiments using cells from different donors, each performed in duplicate.

B) Sesn1, Sesn2 and Sesn3 protein expression in human chondrocytes transfected with siSesn. Representative western blot images are shown. Graphs represent quantification of three different experiments. Data are expressed as mean ±95% CI. Data were analyzed by paired-samples t-test. * = $P < 0.05$ versus siSesnCtrl. Graphs represent quantification of

western blots from three different experiments using cells from different donors, each performed in duplicate. Arrow marks Sesn1 specific band.

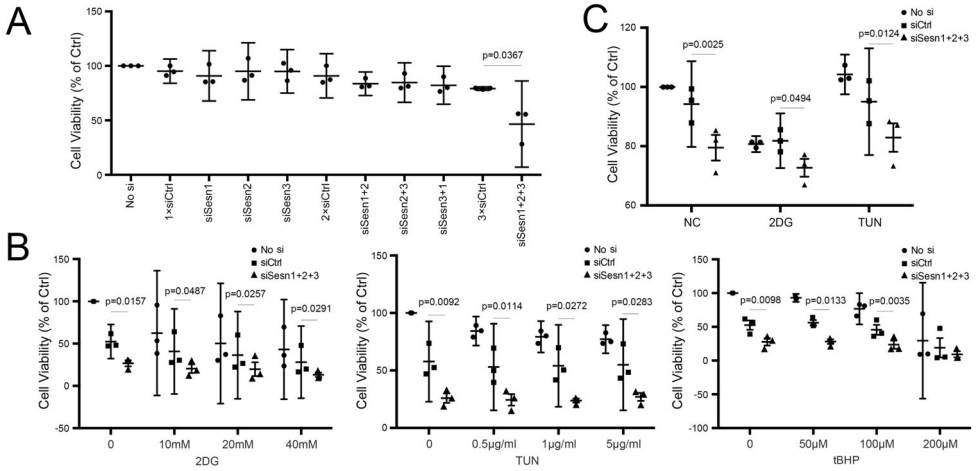

Figure 4. Sesn and chondrocyte viability

A) Cell viability in cultured human chondrocytes transfected with individual and combinations siSesn assessed by MTT assay. Data are expressed as mean ±95% CI. Data were analyzed by paired-samples t-test. * = P < 0.05 versus siCtrl. Graphs represent quantification of MTT assay from three different experiments using cells from different donors, each performed in triplicate.

B) Cell viability in cultured human chondrocytes transfected with siRNA under treatment with different concentrations of 2-DG, TUN and tBHP. Viability was assessed using MTT assay. Data are expressed as mean ±95% CI. Data were analyzed by paired-samples t-test. * = P < 0.05 versus siCtrl. Graphs represent quantification by MTT assay from three different experiments using cells from different donors, each performed in triplicate.

C) Cell viability in cultured human chondrocytes transfected with combinations of siSesn under treatment with 2-DG (20mM), TUN (1ug/ml) and tBHP (250 uM) assessed by Celltiter-Glo assay. Data are expressed as mean ±95% CI. Data were analyzed by paired-samples t-test. * = P < 0.05 versus siCtrl. Graphs represent quantification of MTT assay from three different experiments using cells from different donors, each performed in triplicate.

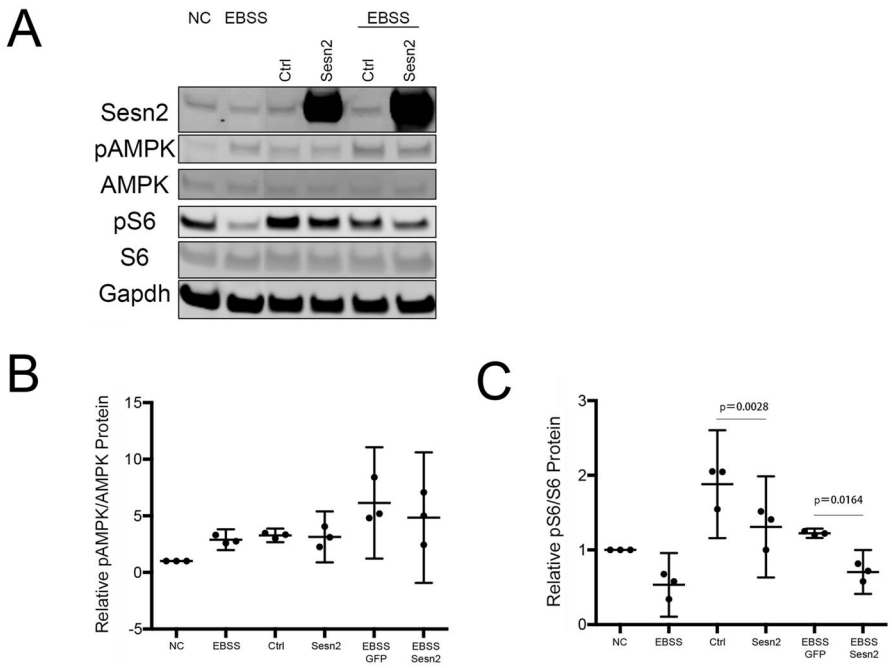

Figure 5. Sesn effects on mTOR and AMPK signaling pathway in human chondrocytes

A) T-C/28 chondrocytes were transfected with indicated constructs for indicated time with or without EBSS and cell lysates were analyzed by western blotting. Representative western blot images are shown.

B) Graphs represent quantification of relative pAMPK/AMPK protein levels by western blots from three different experiments. Data are expressed as mean ±95% CI. Data were analyzed by paired-samples t-test.* = $P < 0.05$.

C) Graphs represent quantification of relative pS6/S6 protein levels by western blots from three different experiments. Data are expressed as mean ±95% CI. Data were analyzed paired-samples t-test. * = $P < 0.05$.

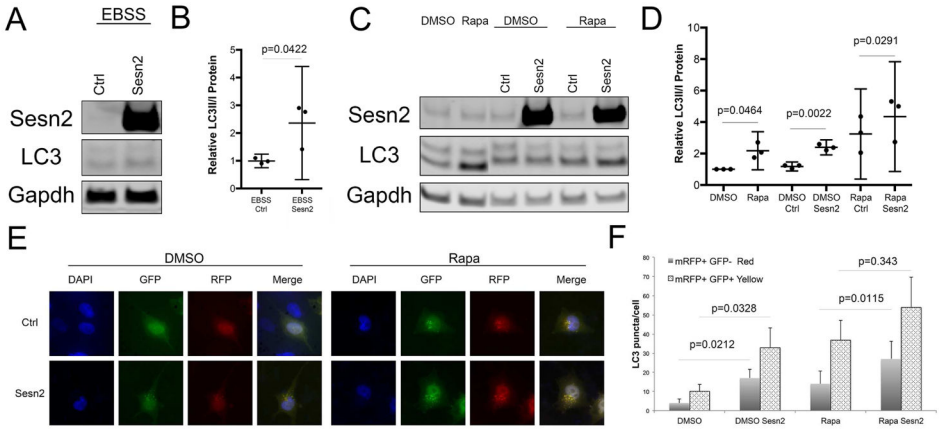

Figure 6. Sesn effects on autophagy in human chondrocytes

A,B) T-C/28 cells transfected with the indicated constructs were treated with EBSS for 1 hour. Western blotting was performed with indicated antibodies. Representative western blot images are shown. Graphs represent quantification of western blots from three different experiments. Data are expressed as mean ± 95% CI. Data were analyzed by paired-samples t-test. * = P < 0.05.

C,D) T-C/28 cells transfected with the indicated constructs were treated with rapamycin (10uM, 6 hours). Western blotting was performed with indicated antibodies. Representative western blot images are shown. Graphs represent quantification of western blots from three different experiments. Data are expressed as mean ± 95% CI. Data were analyzed by paired-samples t-test. * = P < 0.05.

E,F) Autophagosome and autolysosome formation in T-C/28 cells following Sesn2 overexpression. The mRFP-GFP-LC3 plasmid was transfected into T-C/28 cells and 24 hours later cells were transfected with, Sesn2 plasmid. After 24 hours, the cells were treated with rapamycin (10uM, 6 hours). Representative images are shown. Graphs represent quantification of puncta in at least 20 cells from two different experiments. Data are expressed as mean ±95% CI. Data were analyzed by paired-samples t-test. * = P < 0.05.