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Proteasomal function is impaired in human osteoarthritic chondrocytes and promotes decreased SOX9 and aggrecan levels

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Abstract

Objective—Osteoarthritis (OA) chondrocytes have impaired autophagy, one arm of the proteostasis network that coordinates proteome and organelle quality control and degradation. Deficient proteostasis impacts differentiation and viability, and inflammatory processes in aging and disease. Studying OA chondrocytes, we assessed ubiquitin proteasome system proteasomal function.

Methods—We evaluated human knee cartilages by immunohistochemistry, and assessed proteasomal function, levels of proteasomal core subunits and chaperones, and autophagy in cultured chondrocytes. Assays included Western blotting, quantitative RT-PCR, proteasomal protease activity and cell immunofluorescence.

Results—Human knee OA cartilages demonstrated polyubiquitin accumulation, with increased ubiquitin K48-linked polyubiquitinated proteins *in situ*, suggesting proteasomal impairment. Cultured OA chondrocytes demonstrated accumulation of K48 polyubiquitinated proteins, significantly decreased 20S proteasome core protease activity, and decreased levels of phosphorylated FOXO4 and PSMD11, a FOXO4-inducible promoter of proteasomal activation. Levels of proteasomal core subunits PSMB3, 5, 6, and assembly chaperone PSMG1 were not decreased in OA chondrocytes. In normal chondrocytes, PSMD11 siRNA knockdown stimulated certain autophagy machinery elements, elevated nitric oxide (NO) release, and decreased levels of chondrocytic master transcription factor SOX9 protein (and mRNA) and aggrecan (*AGC1*) mRNA. PSMD11 gain of function by transfection increased proteasomal function, raised levels of SOX9-induced *AGC1* mRNA, stimulated elements of the autophagic machinery, and inhibited IL-1-induced NO and MMP13 release in OA chondrocytes.

Conclusion—Deficient PSMD11, associated with less phosphorylated FOXO4, promotes impaired proteasomal function in OA chondrocytes, dysregulated chondrocytic homeostasis, and decreased levels of *SOX9* mRNA, SOX9 protein, and *AGC1* mRNA. Chondrocyte proteasomal impairment may be a therapy target for OA.

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Keywords

Osteoarthritis; Proteostasis; Autophagy; Cartilage; Sox9; Aggrecan; MMP13

INTRODUCTION

OA is a highly prevalent disease of the whole joint, in which aging, biomechanical injury, inflammation, metabolism, and other processes, converge to promote altered articular chondrocyte differentiation and function, and ultimate cartilage failure (1). Multiple hallmarks of tissue aging (2) are detected in OA articular chondrocytes (1). These include deficient activities of cell homeostasis signaling by Forkhead Box O (FOXO) transcription factor and AMP-activated protein kinase (AMPK)(3,4), mitochondrial dysfunction (5,6), and dysregulated proteostasis (5,7–11).

The proteostasis network, which includes the unfolded protein response (UPR), autophagy, and the ubiquitin-proteasome system (UPS), coordinates proteome synthesis and folding, and employs chaperones to direct proteins to loci for degradation (12–14). UPR activation alleviates stress from accumulation of damaged proteins in the ER lumen, but, including in experimental OA, dysregulated UPR activity can contribute to inflammation, tissue damage and degeneration (14–16). Conversely, distinct UPR genes and responses appear decreased in OA chondrocytes (17)

The autophagic process primarily degrades proteins and organelles under hypoxic and low nutrition/energy states, thereby exercising quality control and generating cell energy via recycling (5,7–11). Autophagy is constitutively active in normal articular chondrocytes, promoted by factors including FoxO, AMPK, and regulated in development and DNA damage response 1 (REDD1)(19,20). Autophagy, and AMPK activity, FoxOs, and REDD1, are decreased in aged and OA cartilages (3,4,19,20). Pharmacologic and transgenic inhibition of autophagy potentiates OA in mice (7,8), contrasting with chondroprotective effects of autophagy up-regulation (7,10,11).

The UPS accounts for ~80–90% of cell protein breakdown in proteostasis, via preferential degradation, within proteasomes, of damaged polyubiquitinated proteins, and many short-lived, regulatory cytosolic proteins (eg, I κ B, cyclins)(12,21). In the UPS, isopeptide bonds are formed between ubiquitin and protein substrates by ubiquitin-activating, -conjugating, and ubiquitin-protein ligases (E1–3, respectively)(21). Other ubiquitin molecules are sequentially conjugated to 1 of 7 lysine residues of attached ubiquitin, most commonly at ubiquitin K48, which targets proteins for proteasomal degradation (21). Alternatively, mono- or polyubiquitination (eg, via K63) affects protein function and trafficking, and reduces pressure on the proteasome by stimulating substrate aggregation and removal by chaperone-mediated autophagy (21–25).

The 26S proteasome, composed of 33 distinct subunits, is a cytosolic, energy-requiring ATP-driven "latent proteolytic machine", regulated largely via substrate access, subunit expression, assembly, turnover, and function (12,21). Polyubiquitinated proteins bind a multi-unit 19S "regulatory cap". Substrates are partially unfolded, facilitated by

deubiquitinating enzymes, to enter the barrel-shaped 20S proteolytic core (12,21). The 20S particle has 4 heptameric rings; the two outer ring α subunits regulate substrate access to the proteolytic core, and two inner ring β subunits have chymotrypsin-, trypsin-, and caspase-like activities (21). The 19S cap ATPases stimulate proteolytic activity by opening the 20S “gate” for substrate access (21). Additional regulation is exerted by proteasome-dedicated chaperones and subunits, including PSMG1 (26) and PSMD11 (27–30). The UPS also networks extensively with autophagy (12,13), FOXOs, and AMPK (31,32).

Differing proteostasis demands, aging patterns, and stressors, are linked with variances in UPS composition and activity between tissues, and with age (33). Robust UPS capacity in youth maintains proteome integrity, and inhibits senescence (34). Aging facilitates UPS failure, via accumulated misfolded proteins, and, in some tissues, UPS collapse under stress predates aging (21). UPS failure promotes multiple hallmarks of aging (12,13,21,22,31), senescence and death in cells, disease in multiple tissues, and decreased organism longevity (21).

Prior studies modulating proteasomal function in OA have been limited to experimental rat and mouse knee OA in wild type young animals (35–38). These works assessed nascent disease triggered by surgical knee instability or monosodium iodoacetate, and principally analyzed effects of potent, broad proteasomal protease pharmacologic inhibition using the peptide aldehyde MG132, started prior to OA onset (35–38). MG132 treatment was chondroprotective, with the integrity of proteasomal function appearing to be permissive for OA development and early disease advancement; decreased proteasomal degradation of the NF- κ B inhibitor I κ B was implicated in these effects, by lessening inflammation and cartilage matrix catabolism (35–38). Our study characterized proteasomal dysfunction in human chondrocytes from donors with knee OA. Results elucidated novel potential pathophysiologic impact of chondrocyte proteasomal impairment in established human OA, and a molecular mechanism for selective reversibility of some OA-promoting effects in diseased chondrocytes via improved proteasomal function.

MATERIALS AND METHODS

Reagents

Unless otherwise indicated, all chemical reagents were from Sigma-Aldrich (St Louis, MO), Recombinant human IL-1 β was from Gemini Bio-Products (Sacramento, CA). High-glucose Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and penicillin/streptomycin solution was from Mediatech (Manassa, VA). The MMP13 ELISA kit was from Thermofisher (Waltham, MA).

Human tissue samples and cell culture

All human subject studies were approved by the San Diego VA and Scripps Research Institute IRBs. Human knee chondrocytes were isolated from autopsy donors that were graded macroscopically, as described (6). Here, we studied Grade 0 normal, and Grade I representing cartilage with an intact surface, and Grade IV representing moderate to severe OA, with overt fibrillation (6). There was no pre-selection of donors or donor samples for

any characteristic other than OA or non-OA. Chondrocytes were cultured in Dulbecco's modified Eagle's (DMEM) high glucose medium, with L-glutamine and 100 IU/ml penicillin/100 µg/ml streptomycin (Mediatech), supplemented with 10% fetal calf serum, at 37°C (6). Chondrocytes were studied in first passage, or, where indicated, as primary cells.

Immunohistochemistry

Human knee cartilage paraffin-embedded sections were incubated with primary antibody for 18 h at 4°C. We used rabbit anti-K48-linked proteins (05–1307, EMD Millipore, Temecula, CA), and anti-total ubiquitin (sc-166553, Santa Cruz Biotechnology, Dallas, TX) antibodies. HRP-conjugated secondary antibody employed SuperPicture Polymer Detection Kit (Thermo Scientific), using colorimetric detection with diaminobenzidine (DAB) substrate.

Transfection and expression plasmid construction

Human PSMD11 cDNA was obtained from Addgene.org (pQTEV-PSMD11, ID 31333), amplified by PCR, and subcloned, with and without human influenza hemagglutinin (HA)-tagging at the N-terminal, into pCDNA3.1(+) plasmid vector between XhoI and BamHI sites (primers cited in Supplemental Table 1), and verification by sequencing. Human chondrocytes were cultured in 6-well plates for 16–18 h, then transfected with PSMD11 using XtremeGENE HP DNA reagent (Roche) following manufacturer instructions, for 72h. SDS-PAGE in Bis-Tris gels (Thermo Scientific), and Western blotting of cultured chondrocyte lysates, were done as previously detailed (6). Primary antibodies to PSMD11 (#14303), Caspase-3 (#9662), PARP (#9532) and HA-tag (#3724) were from Cell Signal Technologies (Danvers, MA). Secondary antibodies (1:5000) were HRP-conjugated and detected with ECL reagent (Super Pico or Femto, Thermo Scientific), either using X-Ray film or Gel Doc system (BioRad)

Western blotting

SDS-PAGE of cultured chondrocyte lysates in Bis-Tris gels (Thermo Scientific), and Western blotting, were done as previously detailed (6). Primary antibodies to PSMG1 (#13378), PSMD11 (#14303), FOXO1 (#2880), FOXO3a (#12829), FOXO4 (#2499), phosphorylated FOXO1/FOXO3a (p-FOXO1/p-FOXO3A) (#9464), Sox9 (#82630), LC3/A/B (#12741), p62 (#8025), and HA-tag (#3724) were from Cell Signal Technologies (Danvers, MA). Antibodies to p-FOXO4 (#47278) were from Abcam (Cambridge, UK), and to MMP13 (#3533) from BioVision (Milpitas, CA). Secondary antibodies (1:5000) were HRP-conjugated and detected with ECL reagent (Super Pico or Femto, Thermo Scientific). Quantification of band intensity was performed with ImageStudioLite (LiCOR, Lincoln, NE), with intensity of bands calculated relative to the background from the same membrane.

Proteasome Activity Assay

Proteasome activity assays specifically analyzed chymotrypsin-like activity present at the core proteasome particle, utilizing an AMC(7-amino-4-methylcoumarin)-tagged peptide substrate (Succ-LLVY-AMC), that releases free, highly fluorescent AMC in the presence of protease activity. In brief, cells were lysed at 4°C in PBS, containing 0.5% NP-40, followed by centrifugation at 15,000 rpm at 4°C. After protein level determination, supernatant was

assayed for chymotrypsin-like activity, using a proteasome activity kit from Abcam (ab107921), per manufacturer instructions. To do so, 10 µg aliquots of protein, including samples and positive control from the manufacturer, were assayed in triplicate, with and without proteasome inhibitor MG132 (200 µM), and incubation for 30 min at 37°C in the dark. Output was measured on a fluorimetric microplate reader at T₁ at 350/440 nm (excitation/emission) and T₂ 25 minutes later. The relative fluorescence units (RFU) were determined as followed: $RFU = (RFU_2 - iRFU_2) - (RFU_1 - iRFU_1)$, where iRFU represents wells containing proteasome inhibitor showing non-proteasome activity.

Proteasome activity was calculated as: $Proteasome\ Activity = \left(\frac{B}{(T_2 - T_1) * V} \right) * D$, where: $B =$

Amount of AMC in the sample well (pmol); $V =$ Sample volume added into the reaction well (µL); $T_1 =$ Time (min) of the first reading (RFU₁ and iRFU₁); $T_2 =$ Time (min) of the first reading (RFU₂ and iRFU₂); $D =$ Sample dilution factor. Calibration curves were performed for AMC concentration and purified 20S proteasome activity.

Quantitative reverse transcription (RT)-PCR

RNA was extracted and isolated from cultured human chondrocytes, utilizing RNeasy mini kit (Qiagen, Hilden, GE). RNA levels were quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific); samples with 260 nm/280 nm absorbance ratios >2.0 were used for RT. Equal amounts of total RNA from each sample underwent RT reaction, using Transcriptor High Fidelity cDNA Synthesis Kit (Roche), which includes anchored oligo(dT)₁₈ and random hexamer primers. Quantitative RT-PCR was done using a Roche480 Light Cycler (Roche) and specific primer sets (Supplementary Table 1). Levels of mRNA for genes of interest were calculated as fold change from control or as ratio correlating copy number of gene of interest versus total housekeeping gene GAPDH copy number. For absolute quantification, pGEM-T Easy vector system (Promega, Madison, WI) was used to clone gene fragments (GAPDH, PSMB5, PSMB3, PSMB8 and PSMD11).

Immunofluorescence

Human chondrocytes were cultured on 6-well plates containing round cover slips (12mm, VWR) for 16–18 h, then transfected (XtremeGENE siRNA transfection reagent, Roche) with PSMD11 siRNA (on-target, L-011367-01-0005, GE Dharmacon) for 72 h. Culture media was removed from plates and cells were fixed-permeabilized with cold methanol (–20°C) for 15min. Serial washes were performed with PBS, followed by blocking with 1% BSA in PBS for 2 h at room temperature. Incubation with primary IgG was conducted overnight at 4°C with rabbit anti-LC3A/B (12741, Cell Signal) or rabbit anti-LAMP1 (ab24170, Abcam) or rabbit anti-p62/SQSTM1 (88588, Cell Signal). Secondary antibody incubation (2 h at 22°C) detected primary antibody with Alexa Fluor 488 goat anti-rabbit IgG (A11034, Thermo Scientific) or with Alexa Fluor 555 goat anti-rabbit IgG (21429, Thermo Scientific). Imaging employed a BZ-X700 microscope (Keyence, Itasca, IL).

Chromatin Immunoprecipitation (ChIP) Assay

We tested a defined, specific 48 bp sequence in the Sox9-binding enhancer region of collagen II α1 (*COL2A1*) for cartilage expression (39). We also assessed a defined, functional SOX9/HIF1α complex binding site in the 5'-UTR of *AGC1* (40). To do so, we

used primers corresponding to nucleotides 60–82 [forward] and 220–240 [reverse] in the *AGC1* –640 to –510 promoter region (GenBank: AF031586). ChIP assay (Millipore) was carried out per manufacturer instructions, using Protein A agarose/Salmon Sperm DNA (50% slurry) reduce nonspecific background, and immunoprecipitation with SOX9 antibody (ab3697, Abcam). Following elution of histone/DNA complexes, and DNA recovery by phenol/chloroform extraction, DNA was resuspended in 10 μ L TE buffer. PCR was conducted with 1 μ L of eluate, 2 μ L of 20 μ M primers for Col2a1 and AGCN1 (Supplemental Table 1), 10 μ L KAPA2G Fast ReadyMix PCR (Kapa Biosystems reagent from Sigma-Aldrich) and H₂O up to 20 μ L reaction volume.

Statistical analyses

Statistical analyses were done using GraphPad Prism software. The difference between means of unpaired samples was performed using two-way analysis of variance with Bonferroni's post-test or using unpaired t-test.

RESULTS

Ubiquitinated protein substrates are increased in human OA chondrocytes and OA cartilage *in situ*

We observed increased levels of ubiquitinated proteins in OA compared to normal chondrocytes, in Western blot of cell lysates analyzed for total ubiquitin (Ub), and K48-linked substrates (Figure 1A, B, respectively). Both ubiquitin (Figure 2A) and K48-linked polyubiquitin levels were increased in human knee OA cartilages, compared to normal cartilages, assessed by immunohistochemistry (Figure 2A, B, respectively). In Grade IV OA cartilages, polyubiquitin staining was particularly intense in chondrocyte clusters (Figure 2B).

Decreased chondrocyte proteasome activity in OA

Proteasome activity was significantly decreased in cultured chondrocyte lysates from Grade IV OA compared to normal donors (Figure 3A). Examining three of the seven 20S proteasome core β subunit constituents of the inner ring, where catalytic activity is localized, Western blots revealed no significant decrease in PSMB3, PSMB5 and PSMB6 levels in Grade IV OA chondrocytes (Figure 3B). Hence, we examined the critical proteasome regulatory subunits PSMG1 and PSMD11 (27,28), since PSMG1 facilitates 20S proteasome assembly by promoting grouping of the β subunits into the heteroheptameric α ring (26), and PSMD11 stabilizes the interaction between 19S and 20S subunits of the proteasome (27,28). OA chondrocytes did not have significant decrease in PSMG1 levels (Figure 3C). In contrast, OA chondrocytes demonstrated decreased PSMD11 protein (Figure 4A) and PSMD11 mRNA levels (Figure 4B). Levels of FOXO4, a transcription factor that stimulates PSMD11 mRNA expression (28,31), were grossly comparable in normal and OA chondrocytes. Phosphorylation of FOXOs is required for translocation to the nucleus and subsequent functionality (41). We observed that levels of p-FOXO4 were markedly decreased in OA compared to normal chondrocytes (Figure 4C).

Decreased PSMD11 reduces levels of SOX9 and AGC1 in chondrocytes

We first assessed, via transfection with PSMD11 siRNA, the functional implications of selective decrease of PSMD11 expression in normal chondrocytes. We validated induction of decrease in level of PSMD11, associated with reduced proteasome activity and increased levels of K48 polyubiquitinated peptides (Supplemental Figure 1A–B), without significant changes in levels of total and p-FOXO1, p-FOXO3a, and p-FOXO4 (Supplemental Figure 1C). Under these conditions, in normal chondrocytes, PSMD11 knockdown induced a greater than 70% increase of NO release in response to IL-1 β (10 ng/ml), but no significant difference in MMP-13 protein level (Figure 5A). *MMP3* and *MMP13* mRNA levels were not significantly affected by PSMD11 siRNA (data not shown). In contrast, PSMD11 siRNA knockdown decreased *SOX9* and *AGC1* mRNA levels (Figure 5B), and attenuated SOX9 protein levels in normal chondrocytes (Figure 5C). In normal chondrocytes, PSMD11 siRNA stimulated multiple elements of the autophagic machinery, specifically evidenced by LC3A/B-I to LC3A/B-II conversion and increased levels of the autophagy-UPS adaptor and LC3-binding protein p62 (13) (Figure 5C), with associated perinuclear co-localization of p62 and LC3A/B in normal chondrocytes (Figure 5D).

Effects of PSMD11 gain of function in human OA chondrocytes

Since PSMD11 was decreased in Grade IV OA chondrocytes, we induced selective PSMD11 gain of function by transfection in OA cells. To do so, we followed an approach previously effective in stem cells (28). Transfecting HA-tagged PSMD11 cDNA, we achieved significant increases in proteasome activity, and observed associated decrease in levels of K48 polyubiquitinated substrates in OA chondrocytes (Figure 6A–B), markedly lower IL-1 β -induced NO release (Supplemental Figure 2A), decreased intracellular and released MMP13 (Supplemental Figure 2A–B), and increased SOX9 protein level in 5 of 5 human OA chondrocyte donor samples tested (Figure 6C). Moreover, ChIP assays demonstrated that PSMD11 gain of function increased SOX9 binding to the *AGC1* promoter and *COL2A1* enhancer (Figure 6D). Under these conditions, PSMD11 gain of function stimulated multiple elements of the autophagic machinery, evidenced by changes in LC3A/B conversion and p62 (Supplemental Figure 3A), and associated increase in perinuclear co-localization of p62 and LC3A/B in 5 out of 5 OA chondrocyte donors tested (data not shown). Last, PSMD11 transfection induced increased Sox9 nuclear localization and levels of *SOX9*, *AGC1*, and *COL2A1* mRNA in OA chondrocytes (Supplemental Figure 3B–C).

Discussion

In this study, we demonstrated that proteasomal function is impaired in human knee OA chondrocytes, and that integrity of proteasomal function plays a significant role in maintenance of differentiation of cultured chondrocytes. Our studies included analyses of intersections between proteasomal function and autophagy in normal and OA chondrocytes.

Selective induction of proteasomal dysfunction in normal donor chondrocytes, achieved by PSMD11 siRNA, stimulated multiple elements of the autophagic machinery, including p62 and p62 perinuclear co-localization with LC3. This finding may be due to an acute stress response to PSMD11 knockdown in healthy cells able to adaptively increase autophagy to support proteostasis. Gain of proteasomal function in OA donor chondrocytes, achieved

selectively by PSMD11 transfection, was able to induce increase in elements of the autophagic machinery. Distinct autophagic pathways (microautophagy, chaperone-mediated autophagy, and macroautophagy) effect degradation of cytosolic proteins, employing multiple enzymes, and differing cargo delivery to lysosomes or vacuoles, and with ordered phases of each process (13). Full examination of cross-talk between the UPS and autophagy processes (13), and evaluation of ultimate autophagic protein degradation, were beyond the scope of this study. However, effectiveness of the autophagic process in OA chondrocytes is known to be decreased once OA is well-established (3,4,19,20). As such, our results suggest that proteasomal function compromise is likely more deleterious in aging and OA chondrocytes (12) by a compound proteostasis defect that includes autophagy impairment (7–11). The impact of proteasomal dysfunction in OA chondrocytes could potentially be amplified by decreased autophagic removal of damaged proteasomes (42).

Our study defined molecular mechanistic factors contributing to deficient proteasomal function in human knee OA chondrocytes, and associated accumulation of K48 polyubiquitinated substrates. Specifically, we linked proteasomal dysfunction in OA chondrocytes to decreases in PSMD11 and of the PSMD11 transcription inducer p-FOXO4, but not total FOXO4. PSMD11, a 19S proteasomal subunit, is required for assembly of a functional 20S proteasomal core (27,28,31). Essentially, 20S particles exist as free complexes, with constitutively latent proteolytic activity primed by 19S particle ATPases that also permit access of proteins to the 20S core (27,28). Two members of the forkhead box O family, FOXO1 and FOXO3, are decreased in aging and OA chondrocytes *in vitro*, and articular cartilages *in situ* (3,19). Though FOXO1 and FOXO3 are far more abundant than FOXO4 in human articular chondrocytes, FOXO1 and FOXO3 do not have a defined role in regulating proteasomal activity, unlike FOXO4.

The observed decrease in PSMD11 in OA chondrocytes was at least partially selective, relative to other modulators of proteasome assembly and 20S proteasomal core protease activity. Specifically, 3 inner ring β subunits and the 20S particle assembly chaperone PSMG1 were not decreased in OA compared to normal human knee chondrocytes. Another central finding was that selective PSMD11 gain of function by transfection improved proteasomal function in OA chondrocytes, evidenced by increase in 20S chymotrypsin-like proteolytic activity and decreased K48 polyubiquitinated substrates. PSMD11 is regulated by factors other than FOXO4, including cAMP and PKA signaling, which transcriptionally support PSMD11 expression (30). However, cAMP and PKA signaling have mixed effects on matrix catabolism by articular chondrocytes that would theoretically limit their potential translational utility in increasing proteasomal function in OA chondrocytes.

Selective modulation of PSMD11, beyond effects on proteasomal function, had marked functional impact in normal and OA chondrocytes, with major effects on maintenance of SOX9 and chondrocytic differentiation. We defined affected responses to include levels of SOX9 protein and SOX9 nuclear translocation and binding to the *AGC1* and *COL2A1* promoters, and levels of *AGC1* and *COL2A1* mRNA expression. SOX9 is influenced by multiple signals, including those provided by a variety of growth factors, transcription factors, and epigenetic modifications (43). As such, the net contributions of PSMD11 and

proteasomal function on SOX9, AGC1, and maintenance of articular chondrocytic differentiation, remain to be determined both in early and late OA.

PSMD11-selective modulation of proteasomal function significantly affected IL-1 β -triggered release of NO in both normal and OA chondrocytes, and release of MMP13 by OA chondrocytes. The NO findings carry significance partly due to the capacity of NO to exert both anti-anabolic and pro-catabolic effects on extracellular matrix homeostasis in cartilage, including suppression of proteoglycans and collagen synthesis (44). Moreover, excess NO promotes chondrocyte apoptosis (45,46). NO is generated and degraded by a variety of mechanisms in chondrocytes (46). Moreover, the UPS regulates NO metabolism by complex and paradoxical mechanisms, including regulation of iNOS expression by NF- κ B, and of proteasomal degradation of inducible NO synthase (iNOS)(47,48). Hence, the mechanism of the observed capacity of proteasomal dysfunction to modulate NO release in chondrocytes could be multifactorial.

Both UPS dysfunction and OA increase progressively with aging (1,2,21), and this study was not designed powered to separate effects of aging from OA in chondrocytes. That question clearly will require further study. Another limitation is that our studies of protease activity in isolated proteasomes from cytosol, rather than whole cells, could have readily underestimated the extent of decreased proteasomal function in OA chondrocytes. In this context, substrate selection and gate opening are highly regulated processes that are highly dependent on ATP. OA chondrocytes, largely due to mitochondrial dysfunction, have decreased ATP levels (6).

Our results support a model in which impaired chondrocyte proteasomal function in established OA renders cartilage damage more difficult to slow and potentially reverse. However, there could well be substantially less chondrocyte proteasomal impairment in earlier stages of OA, such as those previously studied (35–38). The chondrocyte UPS dysfunction studied here may reflect an advanced stage of disease, given that we focused on chondrocytes from donors with advanced (Grade IV) OA. Further studies on UPS effects in chondrocytes from cartilages early OA would be of interest. In this regard, multiple pharmacologic proteasome protease inhibitors, including MG132, increase autophagy in normal cells (13,49). As such, our results suggest that chondroprotective effects of MG132 administration in nascent experimental knee OA in young rodents (35–38) may have been partly mediated by induction of increased autophagy in the normal chondrocytes of young animals. Other chondroprotective mechanisms *in vivo* of pharmacologic proteasomal inhibition by MG132 in young animals could include suppression of articular inflammatory processes and cartilage matrix catabolism, and pain (35–38), partially via increased protein stability of the proteasomal degradation-regulated native NF- κ B inhibitor I κ B (37,38). Proteasomal protease inhibitors such as MG132 are only one means to elucidate proteasome function in OA. Specifically, mice transgenic for K48R-mutated ubiquitin, to study focused interference with polyubiquitination, had only marginal and focal chondroprotection in rodent knee instability-induced OA, albeit in a system with potentially problematic residual effects of endogenous normal ubiquitin (38). In the end, questions concerning the impact of the phenotype of proteasomal dysfunction in OA chondrocytes would benefit most from replication of specific molecular mechanisms causing proteasomal function impairment.

This is particularly the case because not only proteasomal core proteases, but also deubiquitinases impart and tune proteasomal functionality (13,21).

In conclusion, we have provided the first evidence, to our knowledge, of impaired proteasomal function in human OA chondrocytes. We mechanistically implicated decreases in chondrocyte PSMD11 and p-FOXO4, with consequent decreases in *SOX9* mRNA and SOX9 protein and *AGC1* promoter and *COL2A1* mRNA levels, along with increased NO release in response to inflammatory stimulation. However, we note that UPS dysfunction promotes disease partly by effects on diverse processes, promoting mitochondrial dysfunction, oxidative stress, and altered IGF receptor, insulin receptor, PI3K, and Akt signaling (12,13,21,22,31). Translationally, our results revealed that proteasomal gain of function, achieved in a highly selective manner by PSMD11 gain of function, stimulated elements of the autophagy process in both normal and advanced knee OA chondrocytes, and also increased *SOX9*, *AGC1* promoter and *COL2A1* enhancer binding, and *AGC1* and *COL2A1* mRNA. Pharmacologic means are emerging to up-regulate 20S proteasomal activity, including by use of small molecules such as deubiquitinase inhibitors (50). The chondrocyte proteasome could provide a novel, pharmacologically targetable therapy in OA, potentially more chondroprotective in well-established disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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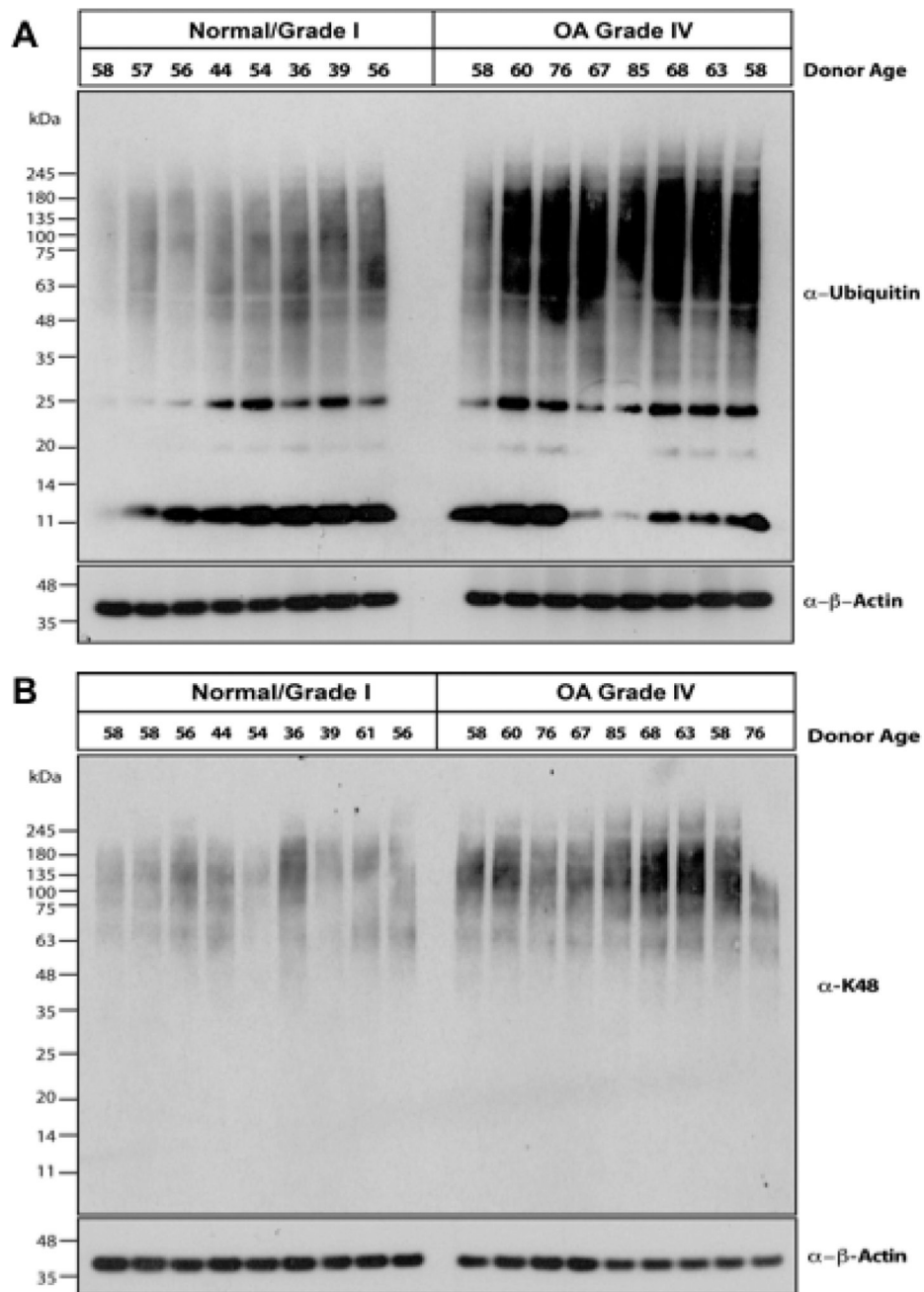


Figure 1. Increased ubiquitin and polyubiquitinated substrate levels in human knee OA chondrocytes

Equal aliquots (10 μ g) of lysates from normal (Grade 0/Grade I) and Grade IV OA human articular chondrocytes were used for 4–12% SDS-PAGE/Western blot analysis, employing antibodies directed against ubiquitin (Ub, n=16) (**A**) and K48-linked ubiquitin (n=18) (**B**). Equal protein loading was also confirmed by blotting for β -actin.

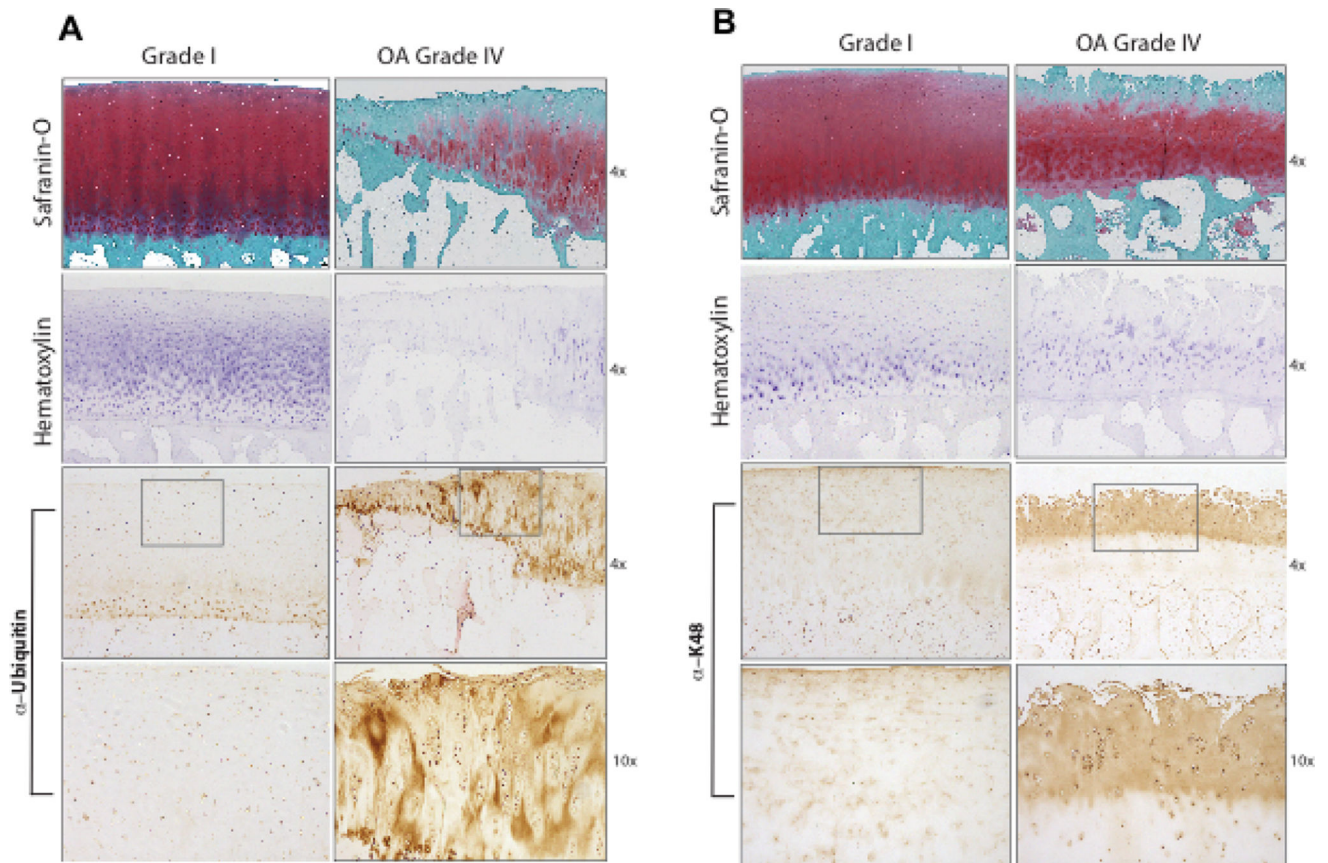


Figure 2. Increased levels of ubiquitin and polyubiquitinated substrates in human knee OA cartilages *in situ*

Immunohistochemistry of paraffin embedded sections from grade IV OA and normal human knee articular cartilages employed antibodies specific for ubiquitin (Ub) (A) and K48-linked ubiquitin (K48) (B). Safranin-O, and hematoxylin cell staining are shown at 4× magnification. For IHC, 4× and 10× magnification results are shown, with the third panels from the top showing a boxed area magnified in the panels immediately below. Representative of 12 separate normal and 12 separate OA donors.

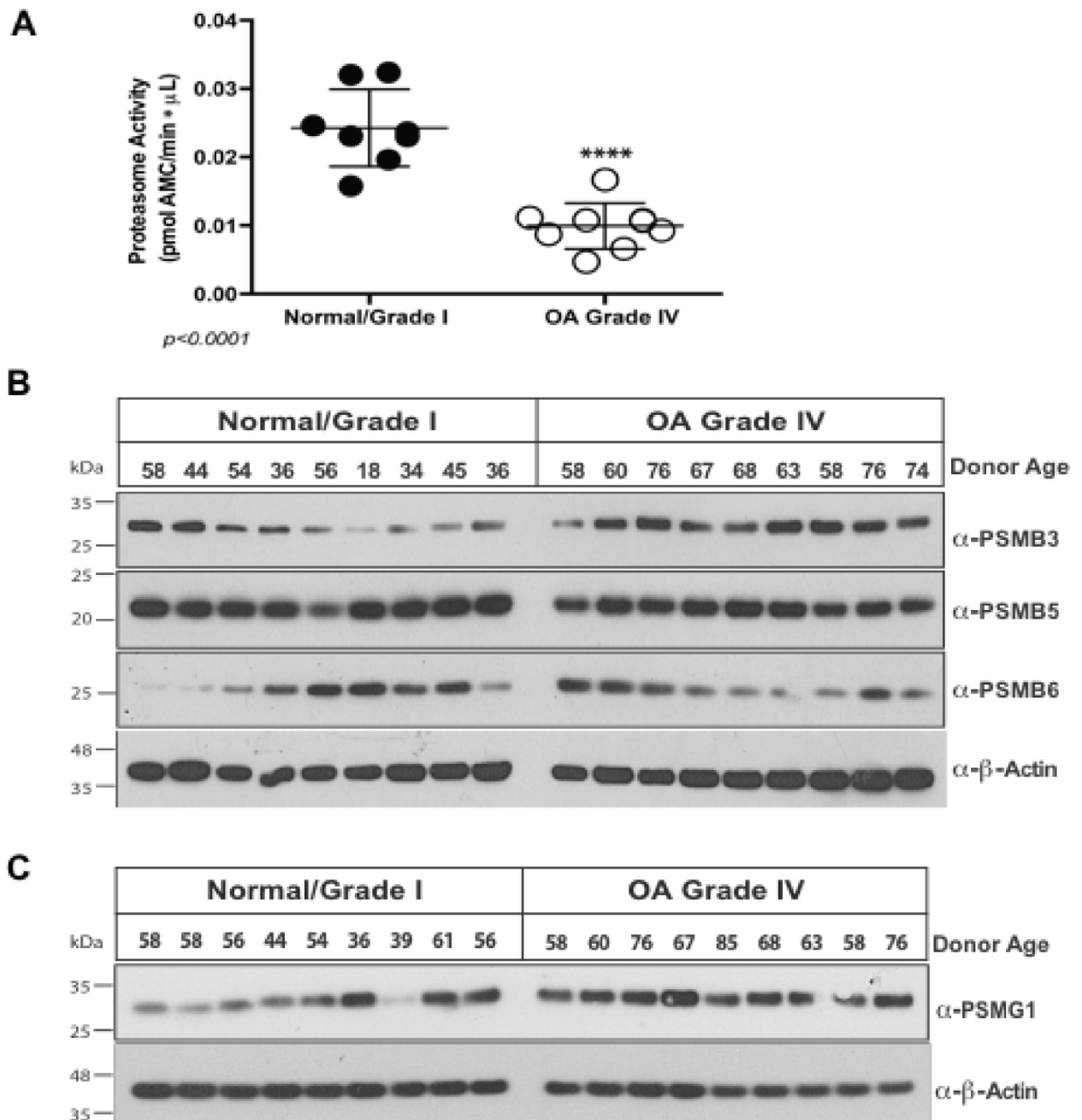


Figure 3. Proteasome activity is diminished in OA chondrocytes, without significant decrease in levels of critical 20S inner ring β subunits and proteasome assembly chaperone PSMG1

(A) Lysates were collected from normal/grade I (n=8) and grade IV (n=8) chondrocytes, in the absence of protease inhibitors; 10 μ g protein aliquots were assayed for 20S proteasome function, using SUC-LLVY-AMC substrate, measuring at 37°C at 351nm/430nm excitation/emission. Replicate controls had proteasome inhibitor MG132 (200 μ M). We observed greater than 50% decrease ($p < 0.0001$, t -test) in proteasome protease activity in OA. (B) Aliquots (10 μ g) of protein extracted from primary chondrocytes (n=18 donors, 9 normal and 9 grade IV) were analyzed by Western blot to determine levels of critical 20S proteasomal inner ring β -subunits, revealing no significant variation between normal and

grade IV. (C) PSMG1 was not appreciably decreased in a panel of OA chondrocytes compared to normal donor cells.

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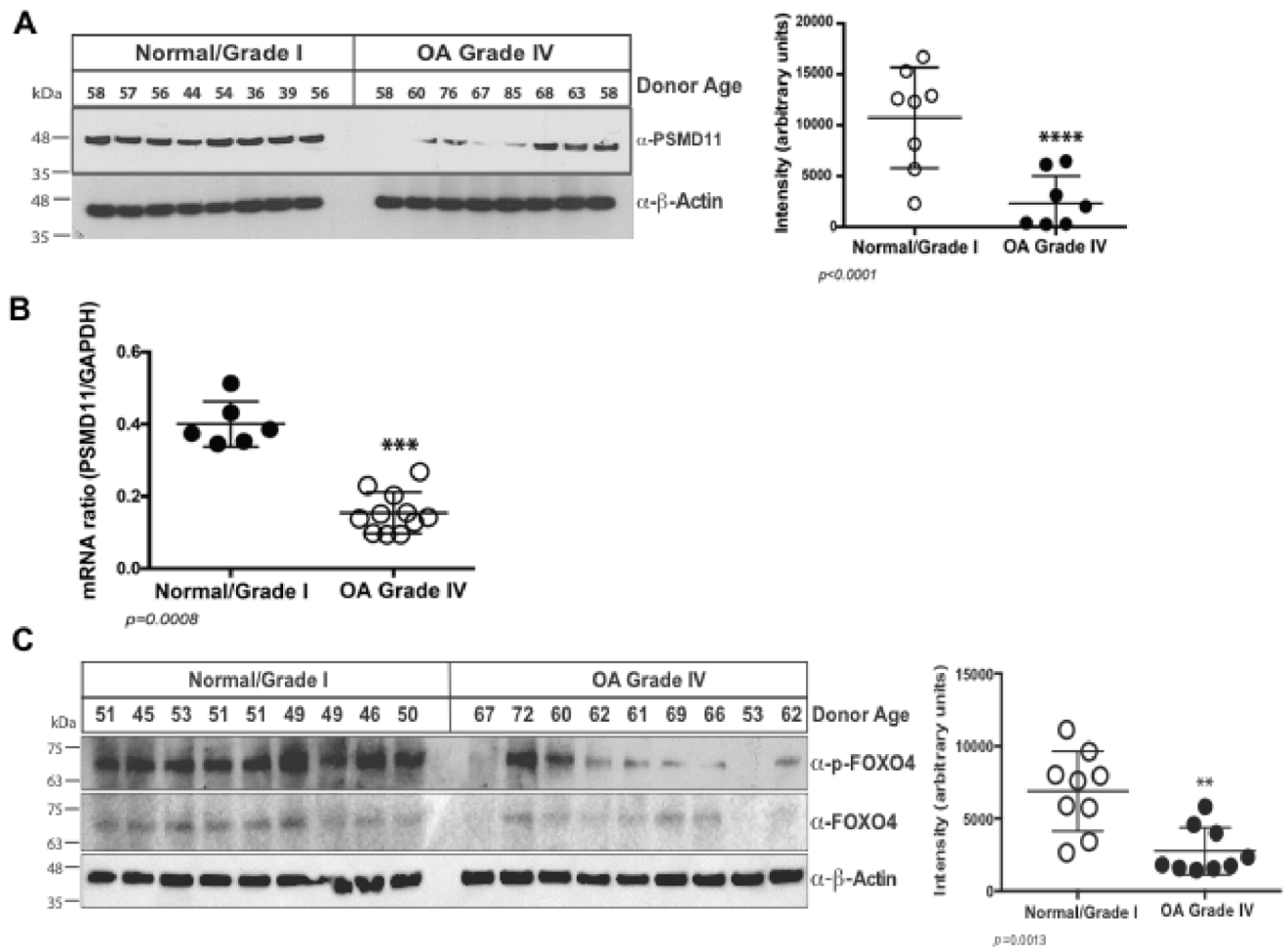


Figure 4. PSMD11 and p-FOXO4 levels are decreased in OA chondrocytes

(A) Western blots of chondrocyte lysates, with densitometric analysis, revealed decreased PSMD11 in OA grade IV compared to normal donor cells. (B) *PSMD11* mRNA levels were significantly decreased in OA compared to normal chondrocytes ($p=0.0008$, t -test). (C) Western blots, with densitometric analysis, also revealed decreased p-FOXO4 levels, but no consistent changes in total FOXO4, in OA compared to normal donor chondrocytes.

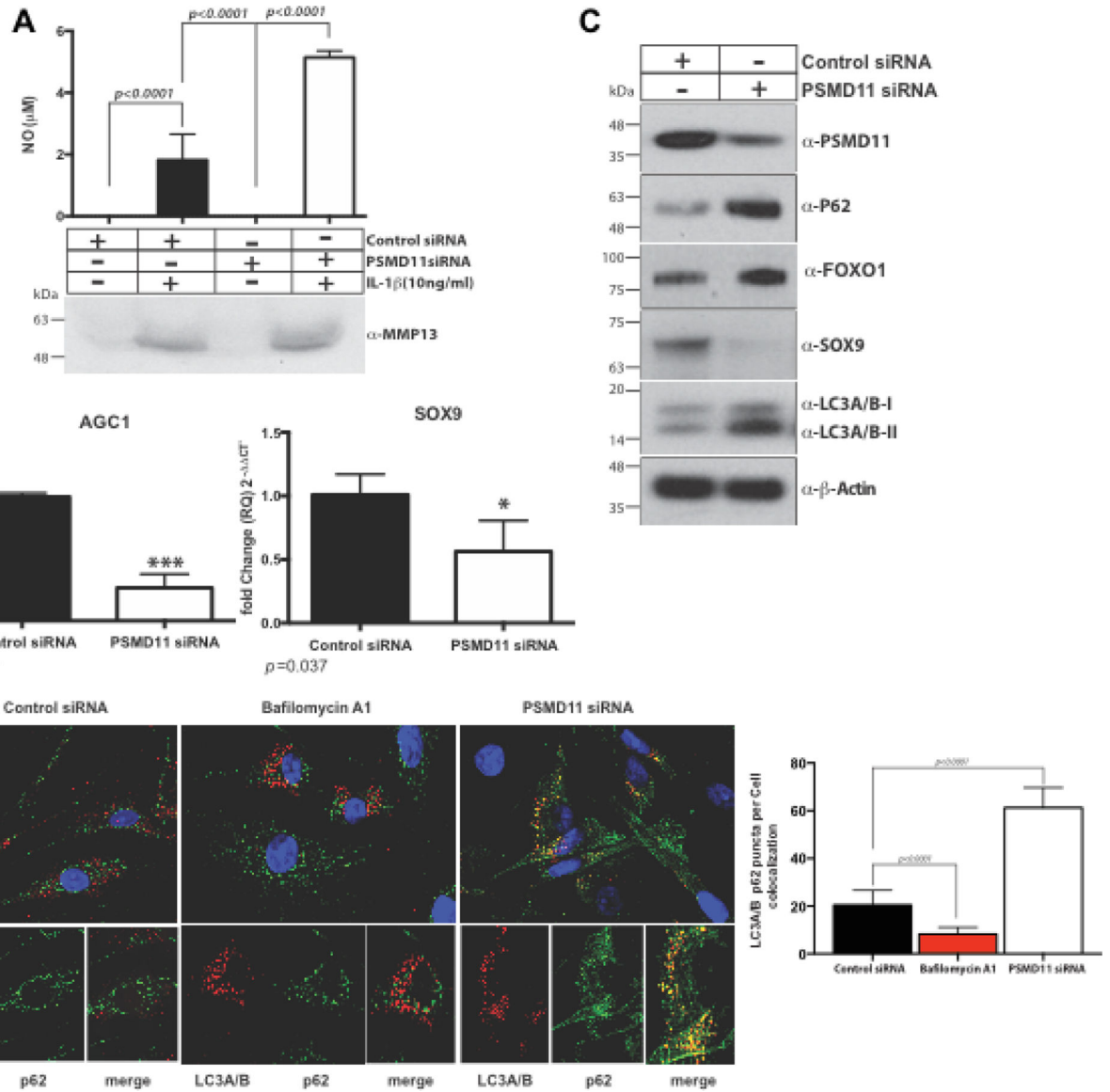


Figure 5. *PSMD11* siRNA knockdown induces increased NO release, induces decreases in *SOX9* and *AGC1*, and stimulates elements of the autophagy process, in normal chondrocytes

We studied *PSMD11* siRNA knockdown effects on NO and MMP13 release in response to IL-1 β (10 ng/ml) in normal human knee chondrocytes (A). We also analyzed *PSMD11* siRNA effects on *SOX9* and *AGC1* (B), and *SOX9* protein expression, as well as autophagy-related LC3A/B-I to LC3A/B-II conversion, the autophagy-UPS adaptor and LC3-binding protein p62 (C), and perinuclear co-localization of p62 and LC3A/B-I to LC3A/B-II in normal chondrocytes (D), with 300 cells/treatment counted to quantify structures where LC3A/B and p62 co-localized. Analysis was by 2-way ANOVA (Multiple comparison with Bonferroni post hoc comparison).

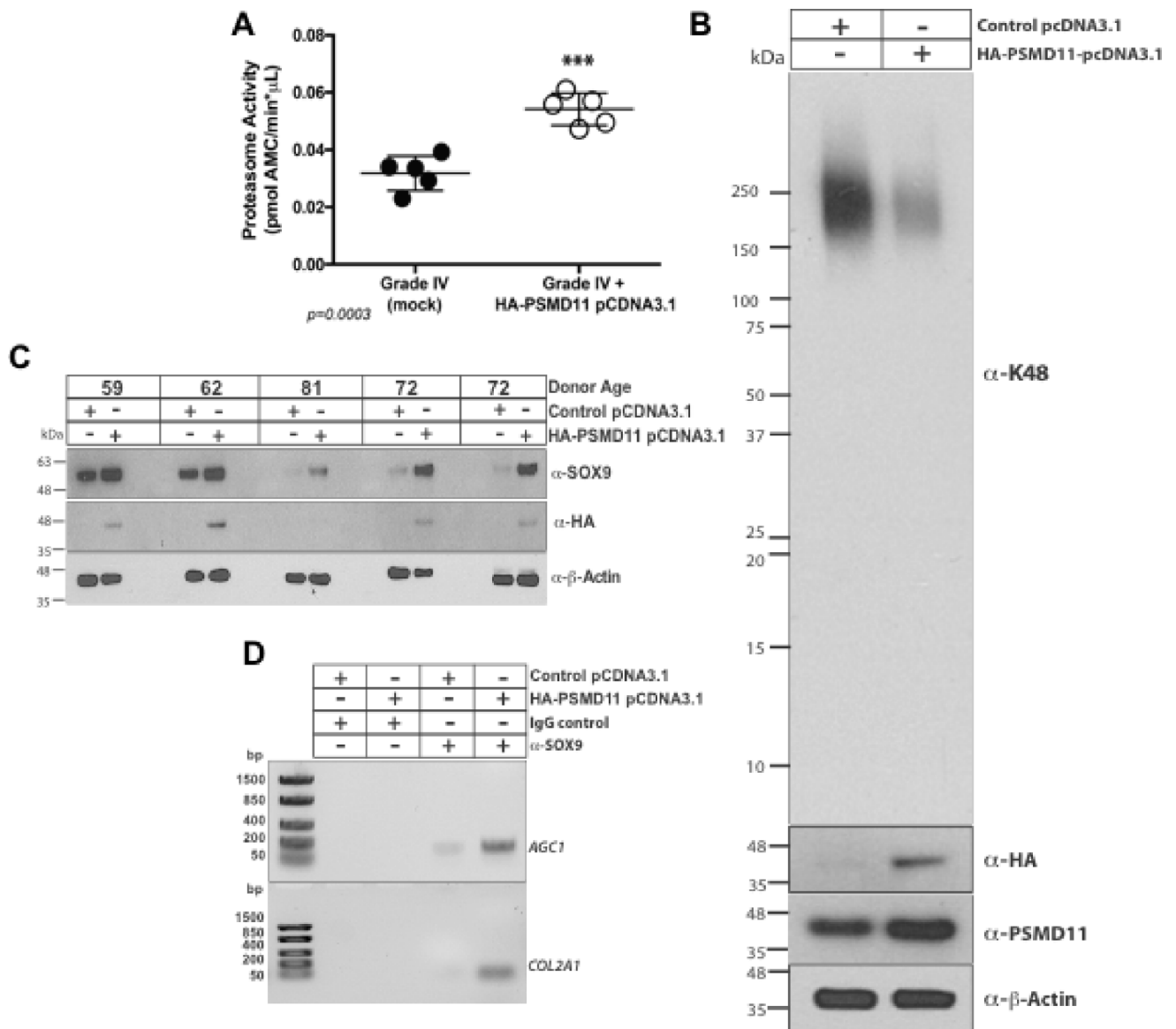


Figure 6. Effects of selective PSMD11 gain of function in cultured human knee OA chondrocytes (A) Transfecting Grade IV OA chondrocytes, we assessed effects of HA tag-*PSMD11* plasmid, compared to control plasmid transfection, on proteasome activity (n=4 donors), and (B) on accumulation of K48-polyubiquitinated proteins (results representative of 4 donors). (C) PSMD11 gain of function by transfection in OA chondrocytes (n=5 different donors), validated to augment cellular HA, was assessed for effects on SOX9 levels. (D) Using ChIP assay, we separately analyzed the effect of PSMD11 gain of function on nuclear SOX9 binding to the *AGC1* promoter and *COL2A1* enhancer.