

Supplementary Information

A thermosensitive, reactive oxygen species-responsive, MR409-encapsulated hydrogel ameliorates disc degeneration through inhibiting the secretory autophagy pathway

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Preparation of PPS-PEG polymer

Synthesis of PEG-Tosyl

A 1.5-g sample of poly(ethylene glycol) methyl ether ($M_w \sim 2$ kDa) was dissolved in 20 mL of a 1:1 (v:v) tetrahydrofuran (THF)/water mixture, then 4 g of sodium hydroxide was added over an ice bath with vigorous stirring. Excess tosyl chloride (4.5 g) dissolved in 15 mL THF was added drop-wise to the above solution. After overnight reaction under ambient conditions, the solution was extracted three times with dichloromethane and distilled water. The transparent viscous liquid PEG-Tosyl was obtained by removing the solvent.

Synthesis of PEG thioacetate

A 0.9-g sample of PEG-Tosyl was dissolved in 20 mL of dichloromethane and 2.28 g of potassium thioacetate dissolved in 20 mL of a 1:1 dichloromethane/MeOH mixture was added. Following addition of 500 μ L trimethylamine, the mixture was incubated for 12 h. The precipitate was filtered and the solvent was removed using a rotavapor. The remaining product was extracted three times with dichloromethane and distilled water. The transparent viscous liquid PEG thioacetate was obtained by removing the solvent.

Synthesis of PEG-PPS diblock polymer

A 200-mg sample of PEG thioacetate was dissolved in 5 mL THF and the solution degassed three times with nitrogen. A 16-mg sample of sodium methoxide in 0.5 mL MeOH was injected into the degassed solution and the mixture left at room temperature for 30 min. Then, 925 mg propylene sulfide was injected into the mixture. After 45 min, 138 mg iodoacetamide was added and the new solution left overnight. The final product was obtained by precipitation with three volumes of ethyl ether.

Supplementary Table1. Patients' disc samples

Characteristics	Degenerated disc group	Non-degenerated
Sample numbers	10	6
Age (years)	56.6 ± 20.1	40.8 ± 7.0
Gender (male/female)	7/ 3	4 / 2
Pfirschmann grade		
I		3
II		3
III		
IV	6	
VI	4	

Note: I–V indicate Pfirschmann grades of discs, listed in Table S5.

Supplementary Table 2. Histological scores of discs.

Histological scores of disc degeneration

I. Cellularity of the annulus fibrosus

Point:

1. Fibroblasts comprise >75% of the cells
2. Neither fibroblasts nor chondrocytes comprise >75% of the cells
3. Chondrocytes comprise >75% of the cells

II. Morphology of the annulus fibrosus

Point:

1. Well-organized collagen lamellae without ruptured or serpentine fibers
2. Inward bulging, ruptured or serpentine fibers in less than one third of the annulus
3. Inward bulging, ruptured or serpentine fibers in more than one third of the annulus

III. Border between the annulus fibrosus and nucleus pulposus

Point:

1. Normal, without any interruption
2. Minimal interruption
3. Moderate or severe interruption

IV. Cellularity of the nucleus pulposus

Point:

1. Normal cellularity with stellar shaped nuclear cells evenly distributed throughout the nucleus
2. Slight decrease in the number of cells with some clustering
3. Moderate or severe decrease (>50%) in the number of cells with all the remaining cells clustered and separated by dense areas of proteoglycans

V. Morphology of the nucleus pulposus

Point:

1. Round, comprising at least half of the disc area in midsagittal sections
 2. Rounded or irregularly shaped, comprising one quarter to half of the disc area in midsagittal sections
 3. Irregularly shaped, comprising less than one quarter of the disc area in mid sagittal sections
-

Supplementary Table 3. Sequences of TRIM16 siRNA.

Genes	sense (5'-3')	antisense (5'-3')
<i>Si-TRIM16#1</i>	GGUGAACAUCAAACUGCAA TT	UUGCAGUUUGAUGUUCAC CTT
<i>Si-TRIM16#2</i>	GCAAUAUUGGACUCCAA TT	UUGGAAGUCCAAUAUUUG CTT
<i>Si-TRIM16#3</i>	CCAUGACUCUGGUUCACAA TT	UUGUGAACCAGAGUCAUG GTT

Supplementary Table 4. Primer sequences in real-time PCR

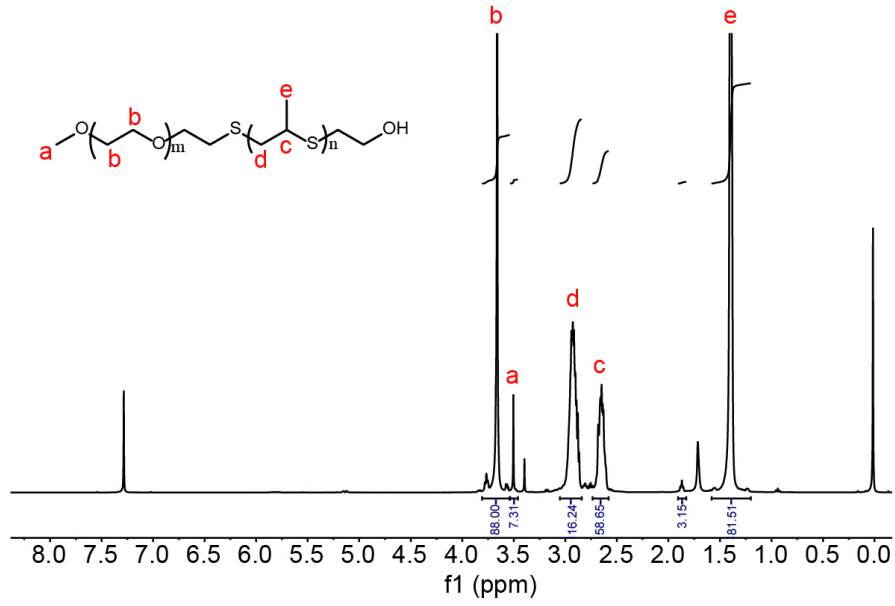
Genes	forward (5'-3')	reverse (5'-3')
Rat SOX9	GACGT GCAAG CTGGG AAG	GTTGG GCGGC AGGTA TT
Rat ACAN	GGCGT CCAAA CCAAC CCGAG	GGAGC TGATC TCATA GCGAT C
Rat ADAMTS5	GCTAC TGCAC AGGGA AGAGG	TGCAT ATTTG GAACC CATT
Rat MMP13	CCATG ATGGC ACTGC TGA	TGGAG CACAA AGGAG TGGTC TCAA
Rat iNOS	TGGGT GAAAG CGGTG TTCTT	TAGCG CTTCC GACTT CCTTG
Rat COX-2	TCCAT TTGTG AAGAT TCCTG TGTTG	TCTCA CTGGC TTATG CCGAA A
Rat TNF-α	ACCAC GCTCT TCTGT CTACT G	CTTGG TGGTT TGCTA CGAC
Rat IL-6	GACTT CCAGC CAGTT GCCTT	GCAGT GGCTG TCAAC AACAT
Rat GAPDH	AGACA GCCGC ATCTT CTTGT	CTTGC CGTGG GTAGA GTCAT

Supplementary Table 5. Pfirrmann disc degeneration score

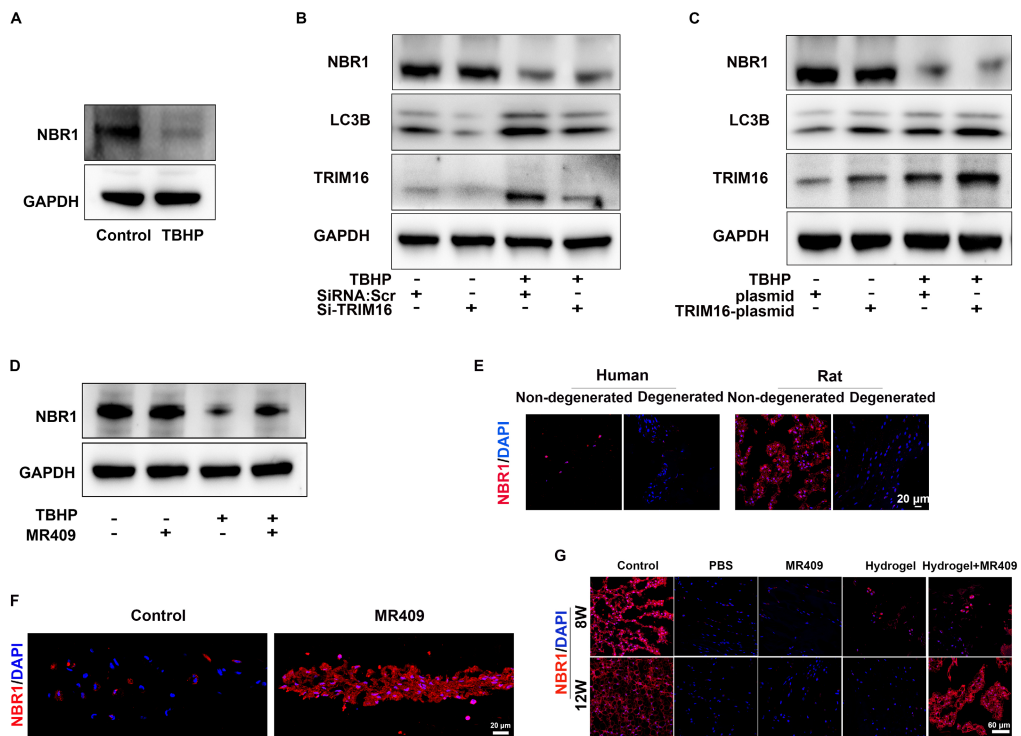
	I	II	III	IV	V
Structure	Homogeneous, bright white	Inhomogeneous with or without horizontal bands	Inhomogeneous, gray	Inhomogeneous, gray to black	Inhomogeneous, black
Distinction of Nucleus and Annulus	Clear	Clear	Unclear	Lost	Lost
Signal Intensity	Hyperintense, isointense to cerebrospinal fluid	Hyperintense, isointense to cerebrospinal fluid	Intermediate	Intermediate to hypointense	Hypointense
Height of Intervertebral Disc	Normal	Normal	Normal to slightly decreased	Normal to moderately decreased	Collapsed disc space

Supplementary Table 6. Sense sequences of ATG5 siRNA.

siRNA	Targeting sequences
<i>ATG5</i> siRNA	5'-GCAACUCUGGAUGGGAUUG-3'

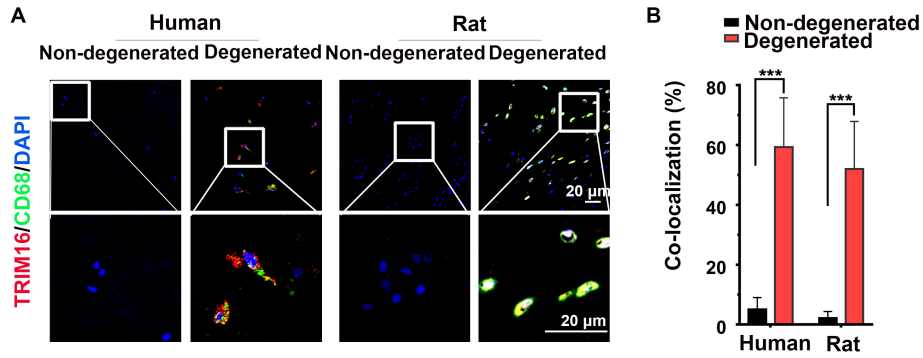


Supplementary Figure 1. ^1H NMR spectrum of the PPS-PEG block polymer.

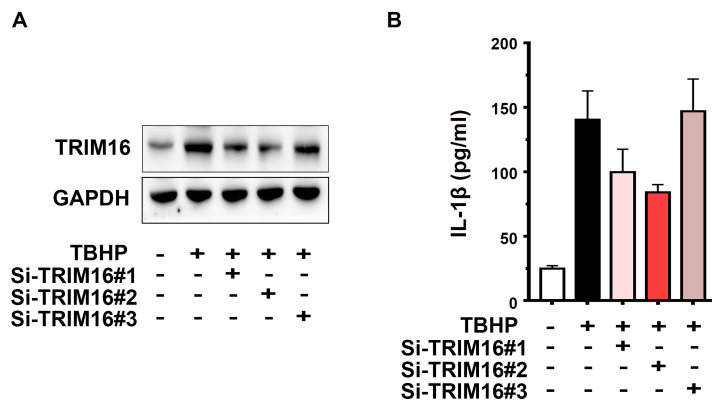


Supplementary Figure 2. (A) Western blot analysis of NBR1 protein expression by THP-1 cells after

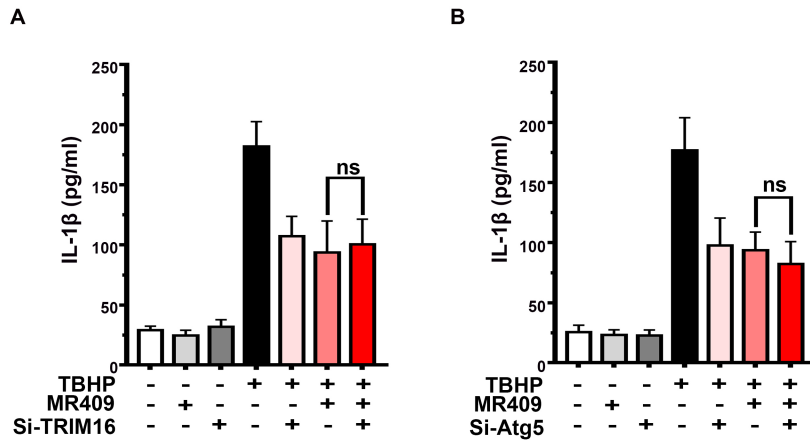
treatment with vehicle or the oxidant tert-butyl hydroperoxide (TBHP) for 2 h. **(B and C)** Western blot analysis of NBR1, LC3B, and TRIM16 protein expression levels by THP-1 cells transfected with TRIM16 si-RNA or TRIM16-plasmid and then treated with vehicle or TBHP. **(D)** Western blot analysis of NBR1 protein expression by THP-1 cells after treatment with vehicle, MR409, TBHP, or TBHP plus MR409 for 2 h. **(E)** Immunofluorescence analysis of NBR1 expression levels in degenerated and non-degenerated discs of human and rat. **(F)** NBR1 immunofluorescence staining in histological sections of the lumbar disc from 23-month-old mice receiving daily subcutaneous injection of vehicle (control group) or MR409 for 8 months. **(G)** Immunofluorescence staining of NBR1 in rat disc sections obtained from the indicated treatment groups at 8 and 12 weeks.



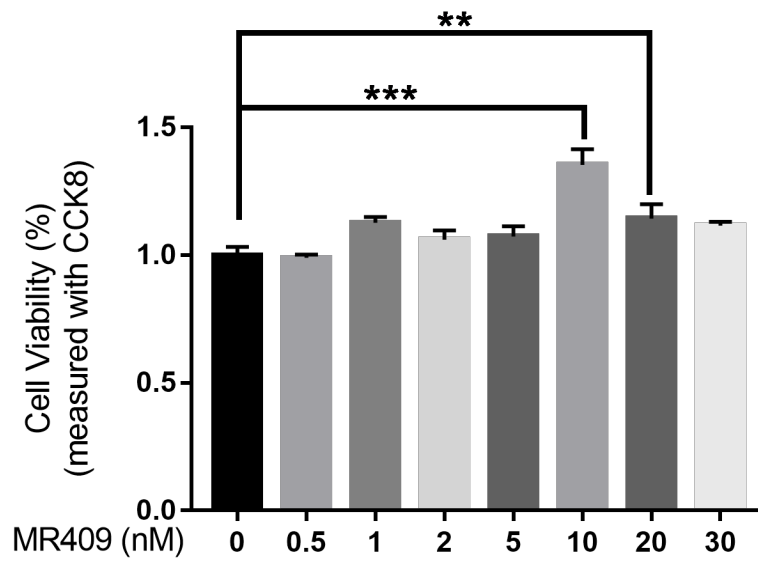
Supplementary Figure 3. (A) Immunofluorescence staining of TRIM16 (red) and CD68 (green) in sections from degenerated and non-degenerated discs of human and rat. (B) Quantitative analysis of immunostaining.



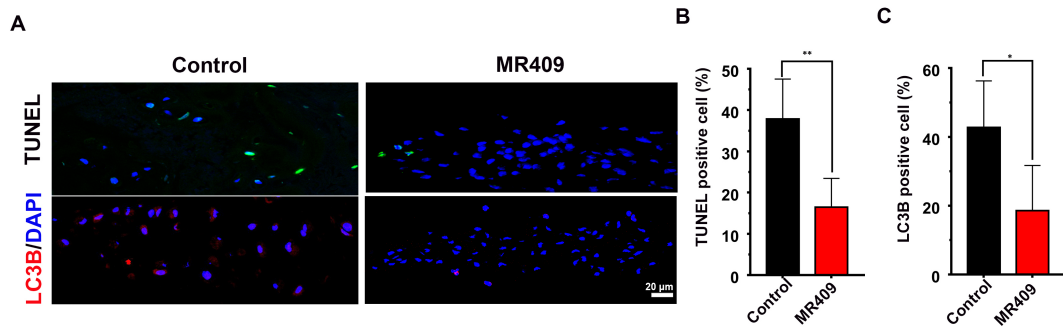
Supplementary Figure 4. (A) Immunoblotting assay of TRIM16 protein expression by THP-1 cells transfected with different TRIM16 siRNAs and treated with TBHP. (B) ELISA assay of IL-1 β in supernatants of THP-1 cells transfected with different TRIM16 siRNAs and treated with TBHP.



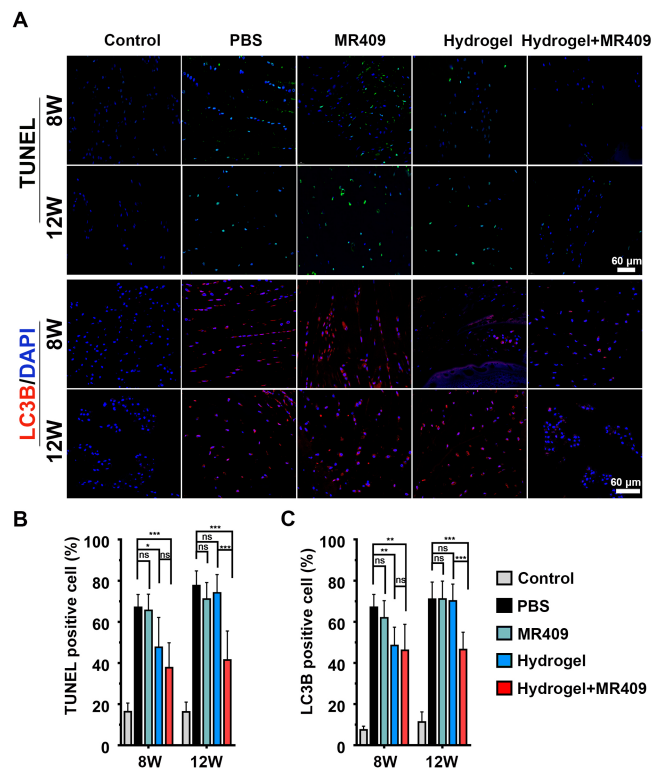
Supplementary Figure 5 A, B. ELISA assay of IL-1 β in supernatants of THP-1 cells treated as indicated.



Supplementary Figure 6. nucleus pulposus cells treated with different concentration of MR409 for 24 h.



Supplementary Figure 7. (A) TUNEL staining of apoptotic cells and immunofluorescence staining of LC3B in lumbar disc sections from 23-month-old mice receiving daily subcutaneous injections of vehicle (control group) or MR409 for 8 months. (B-C) Quantitative analysis of immunostaining.



Supplementary Figure 8. (A) TUNEL staining of apoptotic cells and immunofluorescent staining of LC3B in rat disc sections from the indicated experimental groups at 8 and 12 weeks. (B-C) Quantitative analysis of immunostaining.