p85 Signaling Molecule: A Crucial Component of PGRN/TNFR2 Receptor Complexes, Downregulated in Osteoarthritis and Essential for PGRN/TNFR2-Mediated Anabolic Regulation of Chondrocytes

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Introduction: Despite the high prevalence and morbidity of osteoarthritis (OA), the molecular mechanisms underlying its initiation and progression remain poorly understood. Recently, we reported that activation of anti-inflammatory and chondroprotective TNF receptor 2 (TNFR2) pathway by its ligands, including progranulin (PGRN), a multifaceted growth factor-like molecule, recruited 14-3-3ε to TNFR2 receptor complex and activated ERK signaling and Elk1 transcription factor in chondrocytes and OA (Fu, et al., ***JCI***, 2021; Fu, et al., ***ARD***, 2021). We hypothesized that following the assembly of TNFR2/14-3-3ε receptor complex, additional signaling molecule(s), particularly the kinase and subunits, will be further recruited to this receptor complex to activate ERK/Elk1 signaling pathway in chondrocytes and OA. The current study aims to identify such signal molecule(s) and to determine the importance of the identified molecule(s) in PGRN/TNFR2 regulation of chondrocytes and OA.

Materials & Methods: Biochemical purification followed by mass spectrometry analysis was employed to identify the signal molecule (s) recruited to TNFR2/14-3-3ε receptor complexes in response to PGRN treatment. Co-immunoprecipitation was carried out to validate the interaction between 14-3-3ε and p85 in human C28I2 chondrocytes. CRISPR/Cas9 technology was used to knockout p85 in human chondrocytes. Immunohistochemical staining, western blot and qRT-PCR were used to determine the importance of p85 in regulating chondrocytes metabolism.

Results: Signaling molecule p85 was identified as a component of TNFR2/14-3-3ε receptor complex in response to PGRN treatment. To isolate signaling molecule(s) which were recruited to TNFR2/14-3-3ε receptor complex in response to PGRN treatment, we generated a construct pCMV-Flag-14-3-3ε that expresses Flag-tagged 14-3-3ε, and stable transfection of this construct enable the expression of Flag-tagged 14-3-3ε in 14-3-3ε deficient C28I2 chondrocytes. 14-3-3ε deficient cells transfected with empty vector (serving as a background control) or pCMV-Flag-14-3-3ε was stimulated with or without 200 ng/ml PGRN for 30 min. Flag-tagged 14-3-3ε and its binding proteins were purified by incubations with FLAG affinity columns. The complexes eluted from columns with FLAG peptide were then analyzed by mass spectrometry and MS/MS spectra were searched against the Uni-prot database, using Sequest within Proteome Discoverer. The protein ranking the first was p85 as a novel component of PGRN/TNFR2/14-3-3ε complexes. Co-immunoprecipitation with the lysate of chondrocytes isolated from WT and TNFR2-/- mice was performed to validate the interaction between p85 with 14-3-3ε and TNFR2. The results revealed that p85 was specifically detectable in the immunoprecipitated complex from WT but not TNFR2-/- chondrocytes in response to PGRN treatment, indicating that p85 was associated with TNFR2/14-3-3ε complex in chondrocytes (**Fig. 1a**). In addition, PGRN triggered the activation of p85, evident from the phosphorylation of p85α in response to PGRN treatment in chondrocytes (**Fig. 1b**).

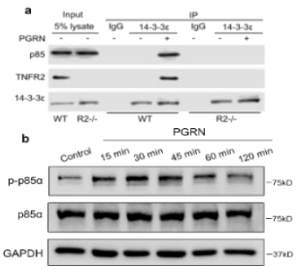

Fig. 1. p85 associates with TNFR2/14-3-3ε complex in response to PGRN in chondrocytes. (a) Immunoprecipitation from PGRN treated WT and TNFR2-/- (R2-/-) chondrocytes with 14-3-3ε antibody and detection of p85, TNFR2 and 14- 3-3ε by immunoblotting. (b) PGRN activates p85α in C28I2 chondrocytes.

p85 is down-regulated in OA articular cartilage. Given that p85 is a downstream kinase activated by PGRN, we further investigated its potential association with the progression of OA. We examined the levels of p85α and phosphorylated-p85α (p-p85α) in human OA articular cartilage, and found that the levels of both p85α and p-p85α were significantly decreased in human OA cartilage compared to non-arthritic healthy cartilage (**Fig. 2a**). In addition, in line with what was observed in human articular cartilage, p-p85α protein levels in mouse articular cartilage were progressively reduced during OA progression compared with the sham group (**Fig. 2b**). These results suggest that the abnormal reduction of p85α in chondrocytes is associated with the progression of OA.

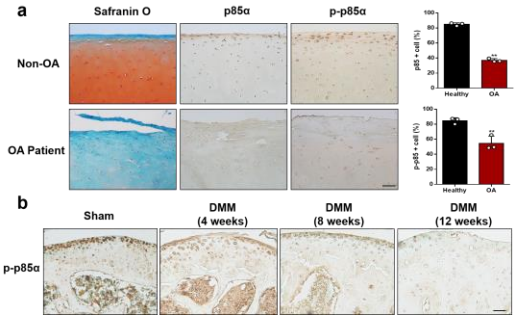

Fig. 2. p85 is reduced in cartilage from human OA and mouse DMM OA model. (a) Safranin O/Fast green staining, and immunohistochemical staining of p85 and p-p85α in Non-OA and OA human cartilage. (b) Immunohistochemical staining of p-p85α in joint section collected from mice subjected to sham or DMM surgery. Scale bar, 50 μm.

Knockout of p85 abolished PGRN-stimulated chondrocyte anabolism through inhibiting ERK and Elk1 activation in human chondrocytes. Following isolation of p85 as a signaling molecule recruited to the TNFR2/14-3-3ε complex by PGRN, we sought to determine whether p85 is involved in the activation of ERK and Elk-1 which are known to transduce PGRN/TNFR2/14-3-3ε signaling to instruct chondrocyte anabolism. First, we generated p85 knockout C28I2 chondrocytes by employing CRISPR-Cas9 technique (**Fig. 3a, b**). Deletion of p85 markedly abolished the activation of ERK and Elk-1 by PGRN (**Fig. 3c**). Accordingly, deletion of p85 blunted the expression of PGRN induced anabolic markers type II collagen (*Col2a1*), aggrecan (*Acan*) and *COMP* (**Fig. 3d**). Collectively, these results indicated that p85 is indispensable for PGRN stimulated chondrocyte anabolism through activating ERK and Elk-1.

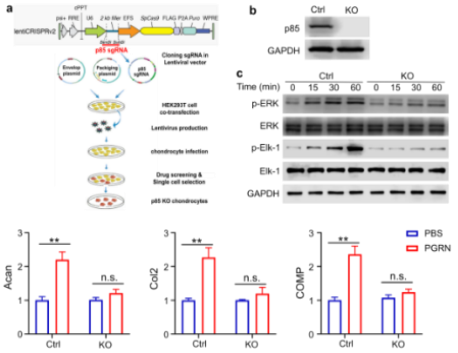

Fig. 3. p85 is indispensable for PGRN regulation of chondrocyte anabolism. (a) Scheme of generating p85 KO chondrocytes using CRISPR/Cas9 technology. (b) Western blotting to confirm the loss of p85 in p85 KO C28I2 chondrocytes. (c) PGRN activation of ERK and Elk-1 is lost in p85 KO C28I2 chondrocytes. (d) mRNA levels of Acan, Col2 and COMP in control and p85 knockout C28I2 cells treated with or without 200ng/ml PGRN for 24hours, assayed by qRT-PCR analysis.

Conclusions: In the current study, we identified signaling molecule p85 as a key component in PGRN/TNFR2 receptor complexes, and found that p85 is downregulated in OA and required for PGRN/TNFR2 regulation of chondrocyte anabolism. In our subsequent study, we will determine the contribution of p85 to mediate PGRN/TNFR2 protective effects against OA *in vivo* using p85 knockout mouse.

Significance: The identification of signaling molecule p85 as a novel component of PGRN/TNFR2 receptor complex not only provides new insight into TNFR2 chondroprotective pathway in orchestrating chondrocyte metabolism, but may also present new target for treating OA and additional cartilage defective/degenerative disorders.

References: 1.Fu, W. *et al. J Clin Invest* 131, doi:10.1172/JCI144016 (2021).

2. Fu, W. *et al. Ann Rheum Dis*, doi:10.1136/annrheumdis-2021-220000 (2021).