Angiogenin Promotes Osteoarthritis Progression via Subchondral Angiogenesis, while a Progranulin-Derived Fragment Mitigates this Effect by Inducing Angiogenin Degradation

Renpeng Zhou, Weirong Hu, Wenyu Fu, Chuan-ju Liu

Department of Orthopaedics & Rehabilitation, Yale University School of Medicine, New Haven, CT, 06519

renpeng.zhou@yale.edu

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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. Subchondral bone changes in OA drive joint degeneration and contribute to cartilage breakdown and pain. 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, activated ERK signaling and Elk1 transcription factor in chondrocytes and OA (Fu, et al., ***JCI***, 2021; Fu, et al., ***ARD***, 2021). Given the comprehensive protective effects of PGRN on OA joints, we hypothesized that, in addition to regulating articular chondrocyte metabolism, PGRN may also influence subchondral bone. This study aimed to identify key molecules that mediate PGRN's regulation of subchondral bone and to assess their significance in the context of PGRN's protective role in OA.

Materials & Methods: The Human Cytokine Array Kit was used to analyze the effect of PGRN treatment on secreted factors from OA chondrocytes. Co-immunoprecipitation validated the interaction between PGRN and Angiogenin (ANG) in human C28/I2 chondrocytes. Tube formation assays assessed the impact of ANG secreted by chondrocytes on HUVEC angiogenesis. ANG levels were measured by immunohistochemistry, ELISA, Western blot to evaluate its expression in chondrocyte. Co-IP is used to detect PGRN-ANG interactions. The destabilization of the medial meniscus (DMM) model was constructed to determine the effect of ANG on OA.

Results: Angiogenin (ANG) is highly expressed in OA articular cartilage and downregulated by PGRN in OA chondrocytes. To identify cytokines secreted by chondrocytes in response to PGRN, we used a human cytokine array to analyze factors from human OA chondrocytes treated with 200 ng/ml PGRN for 72 hours, compared to untreated controls. The analysis highlighted ANG, a protein involved in angiogenesis and cellular stress responses, as having the most significant expression change following PGRN treatment (**Fig. 1a**). The ANG level was increased in human OA cartilage, compared to non-arthritic cartilage (**Fig. 1b**). ELISA and Western blot confirmed that PGRN treatment inhibited ANG expression and secretion (**Fig. 1c, d**). Further, we measured ANG levels in the DMM model and found that ANG expression increased further with the extension of time after DMM surgery, and this increase was more significant in PGRN knockout (KO) mice (**Fig. 1e**).

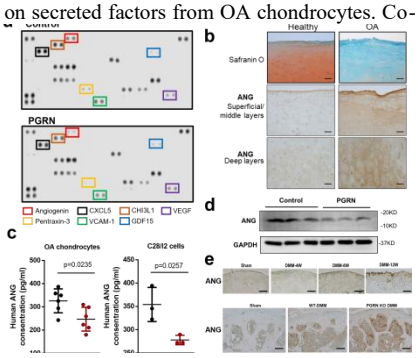

Fig. 1. ANG level is elevated in OA articular cartilage and is negatively regulated by PGRN in chondrocytes. (a) Secretory factors in the culture supernatant of chondrocytes, treated with or without PGRN, were detected using a human cytokine array. (b) Immunohistochemical staining of ANG in healthy and OA human cartilage. (c-d) Detection of ANG expression in human OA and C28/I2 chondrocytes by ELISA and Western blot. (e) Immunohistochemical staining showed the expression of ANG in the DMM model. Scale bar, 50 μm.

PGRN binds to ANG protein and triggers its degradation through the autophagy-lysosomal pathway. Given that ANG expression is negatively regulated by PGRN, we further explored the relationship between PGRN and ANG. Laser confocal microscopy revealed a high degree of colocalization between PGRN and ANG in OA chondrocytes **(Fig. 2a)**. Subsequent Co-IP and molecular docking simulations confirmed an interaction between PGRN and ANG **(Fig. 2b, c)**. By using the proteasome inhibitor MG132 and the autophagy-lysosome inhibitor Baf A1 separately, it was found that PGRN promotes the degradation of ANG through the autophagy-lysosome pathway. **(Fig. 2d)**. Further experiments showed that PGRN binds to ANG via its C-terminal region **(Fig. 2e)**. Using protein purification technology to purify the C-terminal fragment of PGRN that binds to ANG (PGRN-ABF), we observed that PGRN-ABF has a significant inhibitory effect on ANG-induced tube formation in HUVECs. **(Fig. 2f)**. Further experiments have definitively demonstrated that this inhibitory effect stems from PGRN-ABF's ability to promote ANG degradation **(Fig.2g)**.

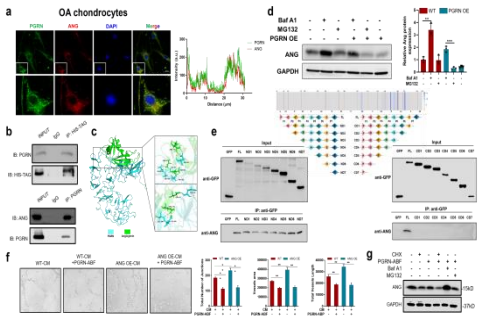

Fig. 2. PGRN accelerates ANG degradation via activation of the autophagy-lysosome pathway. (a) Observation of the co-localization of ANG and PGRN using immunofluorescence staining. (b, c) Co-IP and molecular docking simulating the interaction between ANG and PGRN. (d) Western blot detection of the effects of proteasome and autophagy-lysosome pathway inhibitors on ANG expression. (e) Use CO-IP to verify the specific segment of the binding between PGRN and ANG. (f) The inhibitory effect of PGRN-ABF on tube formation is lost in CM of ANG OE C28/I2 cells. (g) PGRN-ABF promotes ANG protein degradation, which is inhibited by Baf A1.

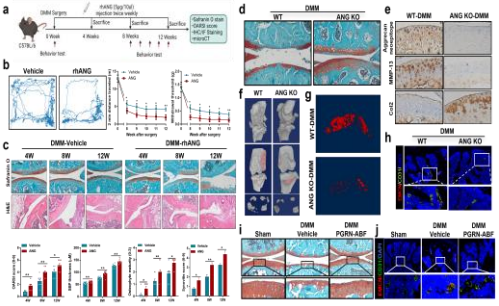

Fig. 3. ANG promotes disease progression in the DMM model. (a) Schematic diagram of the construction of the DMM model and the administration experimental process. (b) Representative traces of open field testing and von Frey test showing the motor ability and pain perception of mice. (c) Analyze the effects of ANG on cartilage and inflammation in the DMM model using Safranin O/Fast Green and HE staining. (d, e) Using Safranin O/Fast green and IHC staining to analyze the protective effect of ANG KO on cartilage in the DMM model. (f, g) Micro-CT detection of osteophytes and subchondral bone vessel volume in mouse joints. (h) Immunofluorescence (IF) detection of H-type blood vessels in mice. (i) Analyze the protective effect of PGRN-ABF on the DMM model using Safranin O/Fast Green staining. (j) IF staining detects H-type blood vessels in subchondral bone.

ANG promotes, whereas PGRN-ABF protects against, OA progression in the DMM model. Next, we explored ANG's role in OA progression. Recombinant ANG (rhANG) was injected into DMM model knee joints **(Fig.3a)**. Behavioral tests showed rhANG significantly increased mouse pain sensitivity and reduced activity range/path **(Fig. 3b)**. Pathological results revealed rhANG disrupted knee bone homeostasis, including cartilage damage and synovial invasion **(Fig. 3c)**. ANG knockout significantly inhibited DMM-induced cartilage destruction **(Fig. 3d, e)**. Micro-CT showed that ANG-KO decreased osteophyte formation and subchondral bone vessel density **(Fig. 3f, g)**; immunofluorescence confirmed reduced H-type blood vessels **(Fig. 3h)**. Additionally, PGRN-ABF injection exerted protective effects similar to ANG KO **(Fig. 3i, j)**.

Conclusions: In this study, we identified ANG as a key downstream secretory factor of PGRN and found that ANG is upregulated in OA, playing a crucial role in PGRN's protection against OA. Our research findings indicate that ANG is essential for subchondral bone angiogenesis in OA joints. In our ongoing study, we are using mice injected with a vascular function inhibitor of ANG to determine the contribution of ANG-mediated angiogenesis to the progression of osteoarthritis and its role in mediating the protective effect of PGRN on osteoarthritis *in vivo*.

Significance: Identifying ANG as a downstream secretory cytokine regulated by PGRN not only highlights its role in angiogenesis but also provides new insights into the role of PGRN in coordinating subchondral bone angiogenesis. At the same time, a fragment that binds to ANG (PGRN-ABF) was discovered and constructed, and it was found that this fragment has a significant protective effect on OA. This finding may reveal new targets and strategies for the treatment of OA and other cartilage-related degenerative diseases.

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).