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Research article

Endothelial RGS12 governs angiogenesis in inflammatory arthritis by controlling cilia formation and elongation via MYCBP2 signaling

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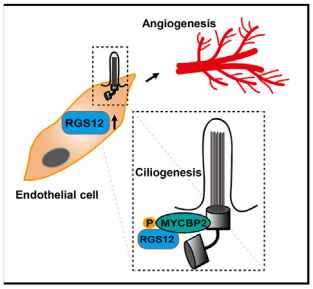
HIGHLIGHTS

- The loss of RGS12 inhibits the development of inflammatory arthritis with the reduction of angiogenesis.
- Endothelial RGS12 promotes cilia formation and elongation.
- RGS12 is associated with the cilia-related protein MYC binding protein 2 (MYCBP2).
- The ciliation of endothelial cells promotes cell migration and angiogenesis and thereby aggravates arthritis.

GRAPHICAL ABSTRACT



ABSTRACT

Angiogenesis is the formation of new capillaries that plays an essential role in the pathogenesis of inflammatory arthritis. However, the cellular and molecular mechanisms remain unclear. Here, we provide the first evidence that regulator of G-protein signaling 12 (RGS12) promotes angiogenesis in inflammatory arthritis through governing ciliogenesis and cilia elongation in endothelial cells. The knockout of RGS12 inhibits the development of inflammatory arthritis with the reduction in clinical score, paw swelling, and angiogenesis. Mechanistically, RGS12 overexpression (OE) in endothelial cells increases cilia number and length, and thereby promotes cell migration and tube-like structure formation. The knockout of cilia marker protein Intraflagellar transport (IFT) 80 blocked the increase in cilia number and length caused by RGS12 OE. Moreover, the results from LC/MS and IP analysis showed that RGS12 is associated with cilia-related protein MYC binding protein 2 (MYCBP2), which enhances the phosphorylation of MYCBP2 to promote ciliogenesis in endothelial cells. These findings demonstrate that upregulation of RGS12 by inflammation enhances angiogenesis by promoting cilia formation and elongation via activation of MYCBP2 signaling during inflammatory arthritis pathogenesis.

1. Introduction

Inflammatory arthritis is characterized by immune cell infiltration of the synovium and angiogenesis. Angiogenesis is a critical process of inflammatory pannus formation. Without angiogenesis, inflammatory cell ingress could not occur (Koch, 2000; Elshabrawy et al., 2015). In patients with inflammatory arthritis, the capillaries and blood-volume fraction were significantly increased compared to the normal tissue (Stevens et al., 1991), and positive correlations were found among the number of synovial blood vessels, infiltration of immune cells, and clinical measurements of synovitis (Rooney et al., 1988).

The vascular endothelium is currently recognized as an essential homeostatic organ that controls the vascular tone and structure. One important function of the endothelium is to sense mechanical (e.g., pressure and shear stress) and/or chemical stimuli (e.g., hormones and locally released vasoactive substances) (Esper et al., 2006). In response to these stimuli, the endothelial cells express and release factors that regulate vasomotor function, inflammatory processes, cell migration, and

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hemostasis. Under the arthritic environment, endothelial cells are stimulated by a large number of inflammatory factors to become dysfunctional and shift the actions of the endothelium toward reduced vasodilation, proinflammatory state, and increased proliferation and migration (Galley and Webster, 2004).

Primary cilia are microtubule-based organelles that enable cells to detect extracellular stimuli and elicit responses (Luu et al., 2018). Recent studies demonstrate that primary cilia are present on vascular endothelial cells, which extend into the lumen of the blood vessel and act as sensors to transmit extracellular signals into the cell. The primary cilia of endothelial cells appear in the early stages of angiogenesis, indicating that cilia involve in early angiogenesis. Conversely, primary ciliary dysfunction on endothelial cells is associated with vascular dysfunctions (Nauli et al., 2008; Ma and Zhou, 2020). Strikingly, studies in osteoarthritis suggest that degenerative and inflammatory condition is associated with the increase in cilia length and prevalence (Mcglashan et al., 2008).

Regulator of G protein signaling (RGS) proteins are important negative molecules of G protein-coupled receptor (GPCR) signaling, which also play essential roles in regulating primary cilia (Yuan and Yang, 2022; Nlend et al., 2002). RGS5 is located in the primary cilia in mouse mesenchymal stem cells and interacts with cilia proteins including acetylated tubulin and SMO (Mahoney et al., 2013). RGS18 regulates the development of cilia in hair cells of the inner ear and neuromast cells (Louwette et al., 2012). Interestingly, we found that RGS12 is also closely related to ciliogenesis in human retinal pigmented epithelium (htRPE) cells by analyzing the RNAi database. Importantly, our previous study has demonstrated that RGS12 is a critical NF-κB enhancer in the development of rheumatoid arthritis (Yuan et al., 2020). In this study, we further investigated the function of endothelial RGS12 in the progression of inflammatory arthritis and identified the potential regulatory mechanisms, which may have potential therapeutic relevance in arthritis.

2. Materials and methods

2.1. Generation of RGS12 $^{fl/fl}$ mice

To generate RGS12-knockout (KO) mice, RGS12 $^{fl/fl}$ mice were crossed with mice expressing Cre recombinase under the control of the tamoxifen-inducible chicken beta actin promoter/enhancer fused to the cytomegalovirus promoter (B6.Cg-Tg (CAG-Cre/Esr1*) 5Amc/J or CAG-CreER; Stock No: 004682, The Jackson Laboratory, US). Recipient mice were injected intraperitoneally with 100 mg/kg body weight tamoxifen daily for five consecutive days (Wang et al., 2016). CAG-CreER control and (CAG-CreER; RGS12 $^{fl/fl}$) KO mice (8 weeks old, n = 10) were littermates derived from breeding heterozygous animals. IFT80$^{fl/fl}$ mice were created for in vitro cilia-related studies as previously described (Yuan et al., 2016). Mice were fed a chow diet and raised in a clean room on a 12-h light/12-h dark cycle. All animal studies were performed in accordance with institutional guidelines and with approval by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania.

2.2. Collagen antibody induced arthritis (CAIA) in mice

CAIA was induced in wildtype (WT) and RGS12 KO mice (C57BL/6 background, 8 weeks old, n = 10) using a mixture of 5 mg of mAbs (Chondrex #53100, US) according to the manufacturer's instructions. Mice in both groups were intraperitoneally injected with 5 mg of monoclonal antibodies (mAbs) on Day 0 and 50 μg of lipopolysaccharide (LPS) (Chondrex #9028, US) on Day 3 to synchronize the development of arthritis (Danks et al., 2016). Mice began to develop arthritis on Day 4 and were euthanized with carbon dioxide on Day 9.

2.3. Clinical scoring of CAIA in the model

Each hind paw of mice was scored on a scale of 0–3 based on the following evaluation criteria: 0, normal; 1, emergence of ankle swelling; 2, moderate to severe redness and swelling of the ankle/wrist/pad; and 3, swelling of the entire paw, including digits, and inflamed limbs with involvement of multiple joints (Crow et al., 2019). The thickness of each hind paw was measured using a thickness gauge and is expressed in millimeters (mm).

2.4. Histological scoring

Hind paws were fixed in 10% formalin, decalcified, and embedded in paraffin. Tissues were sliced into 6 μm sections and stained with hematoxylin and eosin to assess blood vessel numbers and pannus formation scores (synovial membrane hyperplasia and infiltration of leukocytes) with the following modifications: 1 (no detectable change), 2 (slight change), 3 (moderate change), 4 (remarkable change), and 5 (severe change) (Adan et al., 2013).

2.5. Immunofluorescence

Primary endothelial cells were fixed with 4% paraformaldehyde for 10 min at room temperature and incubated with an anti-Ac-tubulin (1:100 dilution, Sigma–Aldrich, US), anti-IFT80 (1:100 dilution, Proteintech, US), anti-RGS12 (1:100 dilution, Sigma–Aldrich, US), anti-CD31 (1:200 dilution, Proteintech, US) antibodies prior to incubation with the appropriate secondary antibody (1:500 dilution; Jackson ImmunoResearch Laboratory, US). The cilia length and ciliated cell numbers were measured using ImageJ software (NIH, US). At least three different mounted preparations were used to acquire images of cells in five fields of view per subgroup at 40 × magnification. All images were visualized on a microscope and acquired with the same exposure time. The relative fluorescence intensity values were determined by comparing each intensity value to the average intensity over the field of view.

2.6. Real-time PCR

RNA from synovial fibroblasts was isolated using TRIzol reagent (Sigma–Aldrich, US) according to the manufacturer's instructions. Then, 1 μg of RNA was reverse transcribed into cDNA using a Reverse Transcription Kit (Takara, Japan). Real-time PCR was performed with a reaction mixture containing primers, the cDNA template, and SYBR Green PCR Master Mix (Bimake, US). The sequences of the real-time PCR primers are as follows: GAPDH (F): AGGTCGGTGTGAACGGATTTG, GAPDH (R): TGTAGACCATGTAGTTGAGGTCA; RGS12 (F): CAGAGTACCCTGCCGAGAAG, RGS12 (R): AGTCTGGGTCCACCATGAAC; IFT80 (F): AGTTATTTGCCGTTGGATCG, IFT80 (R): CCTGCATGGTC CTTCTCTTC.

2.7. Synovial endothelial cells

Primary endothelial cells were isolated from ankle synovium of WT, IFT80 $^{fl/fl}$ and RGS12 KO mice with or without CAIA as described (Abbot et al., 1992; Domer et al., 2021). In brief, synovial vessels (lumen diameter >50 μm) were isolated under the microscope and dissociated prior to gentle rocking for 2 h at 37 °C in endothelial cell culture medium (Lonza, US) containing 0.1% type II collagenase (Sigma, US). The isolated endothelial cells were suspended in phosphate-buffered saline (PBS) containing an anti-CD31 antibody (Proteintech, 11265-1-AP, US) for 30 min and were then washed with PBS and mixed with streptavidin-labeled magnetic beads (Thermo Fisher Scientific,11203D, US). Labeled cells were subjected to magnetic separation and collected by

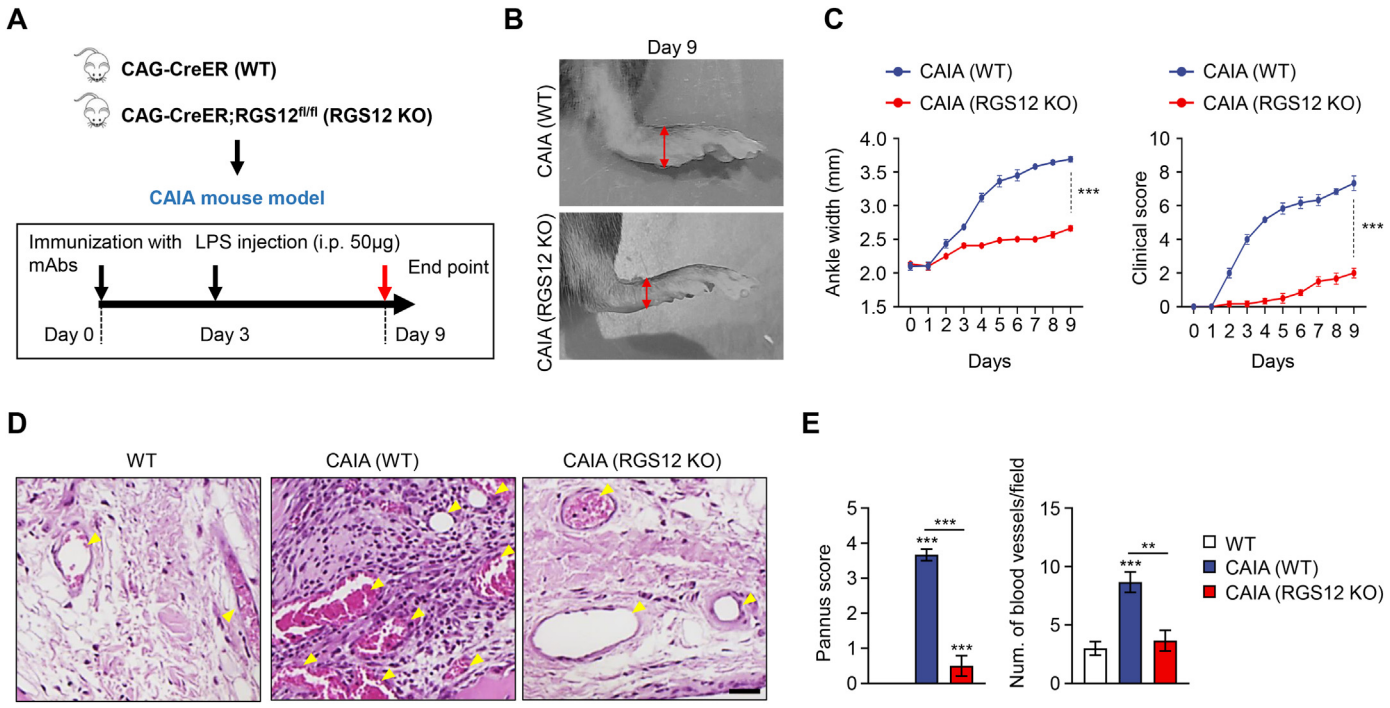

Fig. 1. The knockout of RGS12 inhibits synovial blood vessel formation during inflammatory arthritis pathogenesis. (A) CAIA was induced in CAG-CreER (WT) and CAG-CreER; RGS12 $^{fl/fl}$ (RGS12 KO) mice by injection 5 mg of a mAb mixture (Chondrex #53100) and subsequent challenge with 50 μg of LPS on day 3. After immunization for 9 days, the mice were sacrificed. (B–C) The RGS12 KO mice with CAIA showed decreased ankle widths and arthritis clinical scores in comparison with WT mice. ***$P < 0.001$, n = 10. (D–E) Hematoxylin and eosin staining showed decreased pannus and blood vessels within synovial tissue in RGS12 KO mice with CAIA. Scale bar, 50 mm **$P < 0.01$, ***$P < 0.001$, n = 10.

the addition of endothelial cell culture medium. Endothelial cells were cultured in EGM™-2 Endothelial Cell Growth Medium −2 BulletKit™ (Lonza, US). Cultures were serum starved for 12 h to promote ciliogenesis. IFT80 KO endothelial cells were isolated from the IFT80 $^{fl/fl}$ mice according to the above method and then treated with Ad-Cre (IFT80 KO) or Ad-GFP (Ctrl) as described (Prost et al., 2001). IFT80 deletion in the endothelial cells was confirmed by real-time PCR.

2.8. Cell transfection

Mouse RGS12 (NM_173402.2) cDNA was cloned and inserted into the p3xFLAG-Myc-CMV-26 backbone (Sigma–Aldrich, US). Primary endothelial cells isolated from IFT80 $^{fl/fl}$ mice were seeded on 6-well plates at 3×10^5 cells/well (approximately 90% confluence) and infected with Ad-Cre (IFT80 KO) or Ad-GFP (Ctrl). Cells were transfected with pCMV-vector or pCMV-RGS12 on the following day with Lipofectamine 3000 reagent (Thermo Fisher, US) according to the instructions. Cells were harvested 24–48 h after transfection. For stable transfection, endothelial cells were seeded at 2×10^6 cells per well in a 6-well plate and transfected with pCMV-vector or pCMV-RGS12 plasmids using Lipofectamine 3000 reagent (Thermo Fisher, US) according to the manufacturer's instructions. Forty-eight hours post transfection, cells were treated with 0.4 mg/mL of geneticin (G418, Thermo Fisher, US) every other day for one week until G418-resistant colonies had formed. Stably transfected cells were thereafter maintained in complete medium EGM™-2 Endothelial Cell Growth Medium −2 BulletKit™ (Lonza, US) containing 0.4 mg/mL G418.

2.9. Tube formation

The primary endothelial cells from IFT80 $^{fl/fl}$ mice transfected with pCMV-vector (Control, Ctrl) or pCMV-RGS12 (RGS12 OE) or infected with Cre adenovirus (IFT80 KO) or pCMV-RGS12 + Cre adenovirus (RGS12 OE + IFT80 KO) were resuspended (1.5×10^6 cells/mL) in an endothelial growth medium (EGM-2) supplemented with EGF, hydrocortisone, VEGF, fibroblast growth factor-B, heparin, insulin-like growth factor, gentamicin, and 5% heat-inactivated fetal bovine serum (Lonza# CC3162). For the evaluation of the tube formation, the above endothelial cells were plated on fibrin gel (Millicell, Cat. MMA130, Millipore) coated culture plates. Cells (3×10^5) per well was plated and specific media was added to each group. Tubes formation was accompanied for up to 12 h, and a photographic record at 20x magnification was obtained. Recorded images were then analyzed using the ImageJ software (NIH, USA).

2.10. Migration assay

The endothelial cell migration assay was performed using the Boyden chamber method and 6.5-mm-diameter filters with an 8.0-mm pore size (Corning, US). Briefly, cells were seeded into a Boyden chamber at a final concentration of 6×10^4 cells/mL. Endothelial cell base media (R&D Systems, US) was added to the upper compartments, and endothelial cell growth media supplemented with FBS was added to the lower compartments (R&D Systems, US). The chambers were incubated at 37 °C in the presence of 5% CO_2 for 12 h. Cells on the filters were fixed with methanol for 15 min and stained with 0.1% crystal violet for 15 min. Migration was quantified by counting the stained cells that migrated to the lower side of the filter. The mean number of stained cells per 10 random fields of view was counted for each assay.

2.11. Liquid chromatography–mass spectrometry (LC/MS)

293T cells were stably transfected with pCMV-Flag or pCMV-RGS12-Flag plasmids and cell lysates was used for performing immunoprecipitation by incubating with anti-Flag antibodies. Immunoprecipitated proteins were gel extracted, trypsin digested, and identified by liquid chromatography tandem mass spectroscopy (LC/MS). A stringent set of

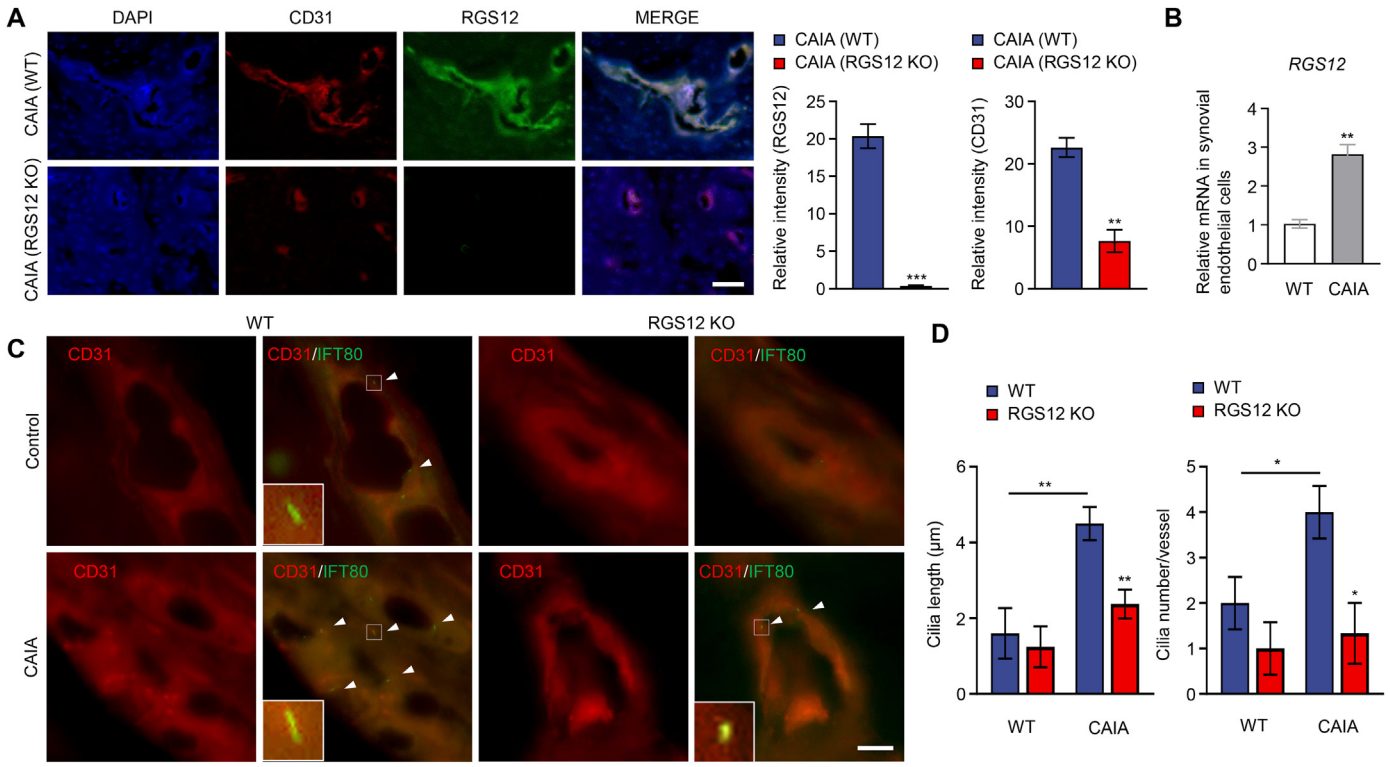

Fig. 2. The loss of RGS12 inhibits synovial angiogenesis and ciliogenesis (A) Immunofluorescence for detecting CD31 and RGS12 expression within synovial vessels from RGS12 KO and WT mice with CAIA. The right panel shows the quantitative date of relative intensity of RGS12 and CD31. Scale bar, 100 μm. The graphs in right panels show the quantitative data on the relative intensity of CD31 and RGS12. $^{**}P < 0.01$, $^{***}P < 0.001$, n = 5. (B) Real-time PCR results showed the increased RGS12 mRNA levels in synovial endothelial cells of CAIA mice. $^{**}P < 0.01$, n = 5. (C) Fluorescence images of synovial tissue in WT and CAIA mice showing CD31-positive vessels (red), and IFT80 stained cilia (green). Scale bar, 20 μm. (D) The cilia number and length per vessel were calculated by immunofluorescence as described in (C). Note that RGS12 KO inhibits the ciliogenesis and cilia length in vessels under the CAIA conditions. Scale bar, 20μm. $^{*}P < 0.05$, $^{**}P < 0.01$, n = 5.

criteria, including a low peptide and protein false discovery rate (FDR) of <0.05, was used for protein identification. Reactome database (https://reactome.org/) was utilized to analyze the protein functions.

2.12. Immunoprecipitation (IP)

Primary endothelial cells isolated as stated above or HEK293 cells (CRL-1573, ATCC, US) were lysed in NP-40 buffer supplemented with a PIC and PMSF (Sigma–Aldrich, US). Briefly, lysates with equal amounts of protein (800 μg) were incubated first with primary antibodies or IgG at room temperature for 1 h and then with protein A/G beads overnight, after which the beads were washed with PBST. Bound proteins were solubilized in loading buffer for Western blot analysis.

2.13. Western blot

Endothelial cells were homogenized with RIPA (radioimmunoprecipitation assay) buffer containing protease inhibitor cocktails (Sigma–Aldrich, US) on ice. Equal amounts of protein (30 μg) were denatured in SDS and separated in 10% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes in transfer buffer containing 20% methanol. The membranes were blocked with 5% skim milk, incubated with primary antibody overnight at 4 °C and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000, Jackson ImmunoResearch, PA) at room temperature for 1 h. β-Actin (1:2000, sc-47778, Santa Cruz) was used as the internal control. The following primary antibodies were used: anti-RGS12 (1:1,000, GW21317, Sigma–Aldrich), anti-p-Tyr (1:1000, Ab10321, Abcam), anti-MYCBP2 (1:1000, ab86078, Abcam), anti-acetylated Tubulin (1:1000, 66200-1-Ig, Proteintech), anti-IκB (1:1000, 10268-1-AP, Proteintech), anti-NFκBp65 (1:1000, 10745-1-AP, Proteintech), and anti-Ubiquitin (1:1000, sc-166553, Santa Cruz).

2.14. Statistical analysis

All data are expressed as the mean ± SEM values. Statistical significance was determined by unpaired two-tailed Student's t-test. Analysis of variance was first performed to compare the mean values among groups, and the Student–Newman–Keuls test was used to compare the mean values between two conditions with GraphPad software 7.0 (San Diego, CA, US). *P* values less than 0.05 were considered significant.

3. Results

3.1. The knockout of RGS12 inhibits the development of inflammatory arthritis and synovial angiogenesis

By analyzing the public RNAi database and Human Atlas database, we found that RGS12 is closely related to ciliogenesis in human retinal pigmented epithelium (htRPE) cells (Fig. S1A), and that RGS12 is highly expressed in the endothelial cells of the cardiovascular system (Fig. S1B). To identify the pathogenesis of inflammatory arthritis, collagen antibody induced arthritis (CAIA) mouse models were established in CAG-CreER wildtype (WT) control and RGS12 knockout (RGS12 KO, CAG-CreER; RGS12 $^{fl/fl}$) mice (Fig. 1A).

To exclude the direct effect of RGS12 deficiency on blood vessels under non-inflammation conditions, we first compared the ankle width and blood vessel numbers between WT and RGS12 KO mice without CAIA and found that there are no significant differences between these two groups (Fig. S2). On day 9 after establishing CAIA mouse models, typical arthritis phenotypes were observed in WT and RGS12 KO mice. The result showed that RGS12 KO mice exhibited less severe phenotypes

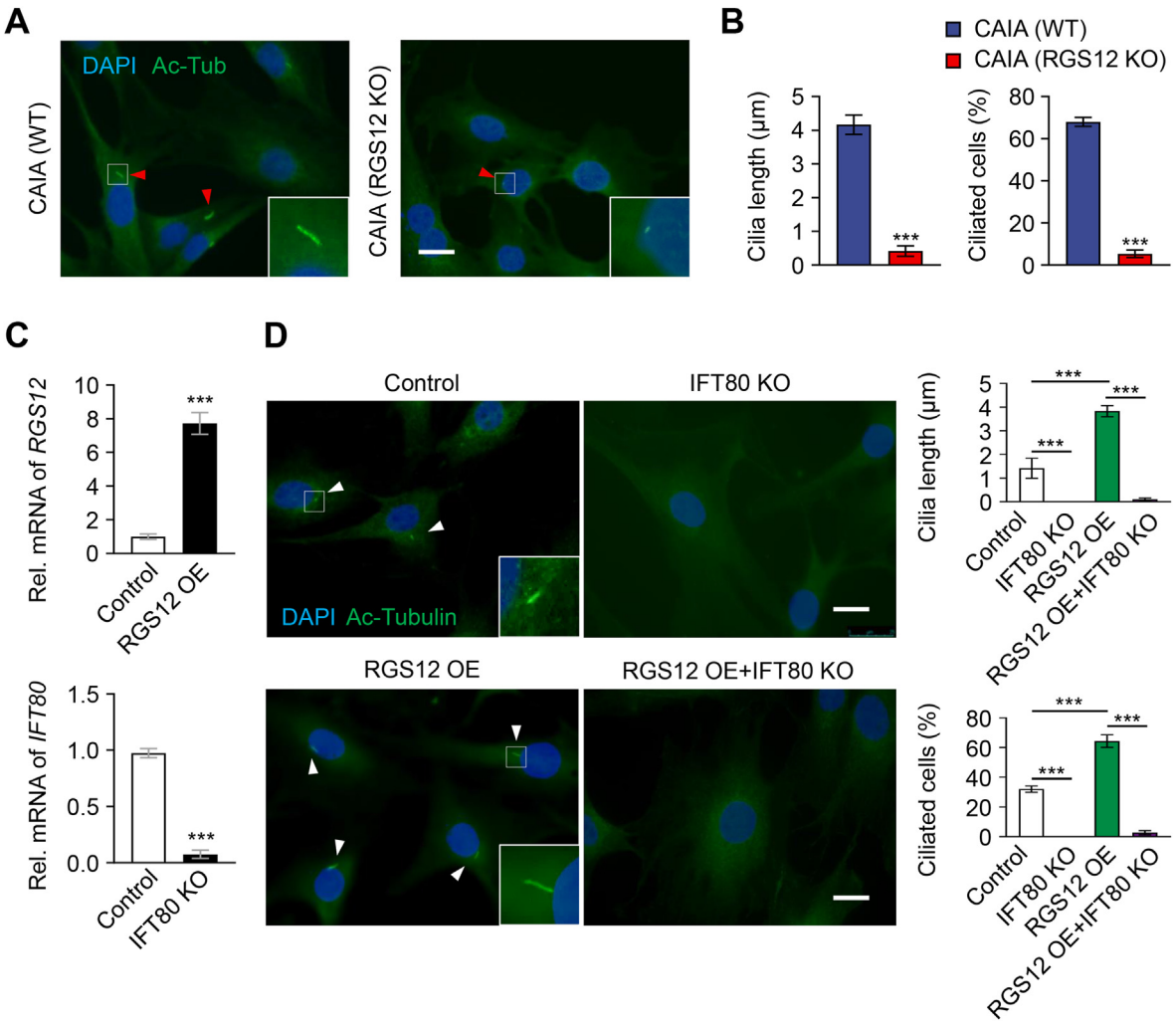

Fig. 3. RGS12 KO leads to the decreased ciliogenesis and cilia length in the endothelial cells from the CAIA mice. (A-B) Immunofluorescence assay for identifying cilia using anti-acetylated-Tubulin (Ac-Tub, green), and DAPI (nuclei, blue). The endothelial cells ($CD31^+$ cells) were isolated from the synovium of WT and RGS12 KO mice with CAIA. Scale bar 20 μm. Note that the ciliated cell numbers and cilia length of synovial endothelial cells were decreased in RGS12 KO mice with CAIA. $^{***}P < 0.001$, n = 10. (C) The primary endothelial cells from IFT80 $^{fl/fl}$ WT mice transfected with pCMV-vector (Control, Ctrl) or pCMV-RGS12 (RGS12 OE), and then infected with adenovirus (Ad)-GFP (control) or Ad-cre to delete IFT80 (IFT80 KO, or RGS12 OE + IFT80 KO). Realtime PCR analysis of RGS12 and IFT80 mRNA levels in WT control, RGS12 OE, and IFT80 KO endothelial cells. The data are presented as the mean ± SEM, n = 5. $^{***}P < 0.001$. (D) The cilia number and length in those cells as described in (C) were determined by immunofluorescence (green, anti-acetylated-Tubulin, blue DAPI nuclei). Note that RGS12 OE in synovial endothelial cells increases the ciliogenesis and cilia length, whereas IFT80 KO inhibits the increased cilia number and length induced by RGS12. Scale bar, 20 μm. $^{**}P < 0.01$, $^{***}P < 0.001$, n = 5.

evidenced by decreased ankle width and clinical score than WT mice with CAIA (Fig. 1B and C). Most interestingly, histological analysis showed that the pannus score and the number of blood vessels were significantly decreased in RGS12 KO mice compared to WT mice with CAIA (Fig. 1D and E).

3.2. *The loss of RGS12 inhibits the synovial angiogenesis and ciliogenesis*

To confirm the histological findings, we further performed the Immunofluorescence (IF) assay. The result showed that both CD31 (endothelial cell marker) and RGS12 were expressed in the blood vessels, and the number of CD31 positive cells was significantly decreased in RGS12 KO mice with CAIA compared to the control mice with CAIA (Fig. 2A), suggesting that loss of RGS12 inhibits angiogenesis during inflammatory arthritis. It is known that the expression of RGS12 was increased in inflammatory conditions (Yuan et al., 2020). To determine RGS12 levels in the endothelial cells of CAIA mouse models, we performed real-time RT-PCR analysis in the endothelial cells isolated from the synovial tissues of WT and CAIA mice. The results showed that RGS12 expression was significantly upregulated in endothelial cells of CAIA mice compared to that in WT mice (Fig. 2B). These findings suggest that RGS12 promotes angiogenesis during the pathogenesis of inflammatory arthritis.

Primary cilia on the endothelial cells are required for vascular homeostasis and the early stages of angiogenesis (Ma and Zhou, 2020). Cilia are also important sensing organelles, showing increased cilia length and prevalence under inflammatory conditions such as Interleukin-1 (IL-1) exposure or in inflammatory diseases (Wann and Knight, 2012). To determine cilia changes in the synovial blood vessels, we performed the immunofluorescence staining for detecting the blood vessels (CD31) and cilia (IFT80). The results showed that cilia length and number in blood vessels were both increased in CAIA mice but significantly decreased in the RGS12 KO mice with CAIA (Fig. 2C and D). Moreover, we found the RGS12 KO decreased IFT80 protein levels in the blood vessels but did not affect the transcriptional levels (Fig. S3).

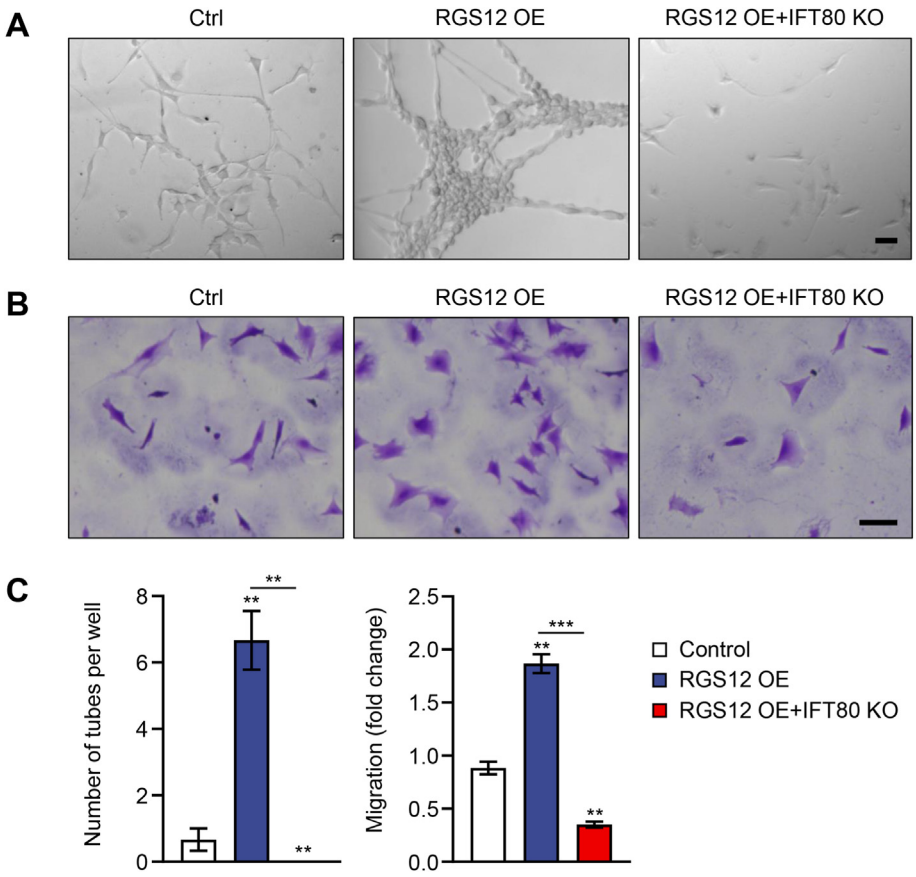

Fig. 4. The overexpression of RGS12 increases angiogenesis and cell migration of wildtype (WT) endothelial cells but has no function in IFT80 deficient endothelial cells. (A-B) Determination of the impact on angiogenesis (tube formation) and cell migration (transwell assay). The primary endothelial cells from IFT80 $^{fl/fl}$ WT mice transfected with pCMV-vector (Control, Ctrl) or pCMV-RGS12 (RGS12 OE) and further infected with Ad-GFP (Ctrl, RGS12 OE) or Ad-cre (IFT80 KO, RGS12 OE + IFT80 KO). Scale bar, 100μm. (C) Quantitative analysis of the tube formation and cell migration were detected as shown in the left panels. Note that RGS12 OE increased the tube formation and cell migration in WT which were blocked by IFT80 KO in endothelial cells. $^{**}P < 0.01$, $^{***}P < 0.001$, n = 10.

3.3. RGS12 KO leads to the decreased cilia length and formation in the endothelial cells from the CAIA mice

To confirm whether cilia were affect by RGS12 KO in the endothelial cells, we harvested the primary endothelial cells from the WT and RGS12 KO mice with CAIA. Consistently, RGS12 KO led to decreased ciliated cell numbers and cilia length in endothelial cells of CAIA mice, suggesting that RGS12 increases ciliogenesis (Fig. 3A and B).

Intraflagellar transport (IFT) 80 is a cilia marker protein that is located in cilia and is essential for cilia assembly and maintenance. We found that knockout of IFT80 (IFT 80 KO) leads to the loss of cilia in endothelial cells (Fig. 3C and D). To further confirm the functions of RGS12 on ciliogenesis, we isolated the synovial endothelial cells from WT and IFT80 KO mice respectively and overexpressed RGS12 (RGS12 OE) in these cells (Fig. 3C and D). The results showed that RGS12 OE in endothelial cells can significantly enhance ciliogenesis and cilia length, which were markedly inhibited by IFT80 KO (Fig. 3D), suggesting that RGS12 acts upstream of cilia regulation.

Cilia are essential for cell migration and angiogenesis (Ma and Zhou, 2020). We hypothesized that RGS12 may control cell migration and angiogenesis by regulating ciliogenesis. To test this hypothesis, the endothelial cells from WT control, IFT80 KO, RGS12 OE, and RGS12 OE + IFT80 KO groups were used for performing the tube formation and transwell assays (Fig. 4A and B). As expected, RGS12 OE in the endothelial cells promoted tube formation and cell migration, which were significantly inhibited by IFT80 KO (Fig. 4A–C).

3.4. RGS12 regulates ciliogenesis via regulating MYCBP2 signaling

RGS12 acts as a scaffold protein to enhance protein activity through posttranslational regulation (Yuan et al., 2020). To further understand the mechanism of RGS12 in regulating ciliogenesis and angiogenesis, we performed the Liquid chromatography/mass spectrometry (LC/MS) experiments (Fig. 5A). 293T cells were stably transfected with pCMV-Flag or pCMV-RGS12-Flag plasmids and cell lysates were used for performing immunoprecipitation (IP) by incubating with anti-Flag antibodies. Immunoprecipitated proteins were gel extracted, trypsin digested, and identified by LC/MS. A total of 235 unique proteins were identified with a protein False Discovery Rate equal to or lower than 1%. The top five unique proteins including U2SURP, MYCBP2, MUC16, ABCF2, and BAG3 were indicated in Fig. 5A.

We then performed the Venn analysis to identify the overlapping proteins between the RGS12 binding proteins and cilia proteins from the CiliaCarta database (https://tbb.bio.uu.nl/john/syscilia/ciliacarta/). We found that MYC-binding protein 2, E3 Ub protein ligase (MYCBP2), a critical ciliary protein, was the most significantly changed protein that associates with RGS12 (Fig. 5B). Consistent with the LC/MS data, Immunoprecipitation (IP) experiment further confirmed the association between RGS12 and MYCBP2 in endothelial cells (Fig. 5C).

MYCBP2 is an E3 ubiquitin (Ub) protein ligase which locates in cilia and maintains cilia functions (Shearer and Saunders, 2016). Consistently, we found the knockdown of MYCBP2 led to the cilia loss in endothelial cells (Fig. S4), suggesting that RGS12 may regulate cilia formation and elongation by regulating MYCBP2. To get further insight into RGS12 regulation in MYCBP2, we performed the IP experiments and found that RGS12 increased the level of MYCBP2 phosphorylation and the expression of cilia marker protein acetylated-Tubulin (Ac-Tub) in endothelial cells (Fig. 5D). Conversely, RGS12 KO leads to the decreased expressions of MYCBP2 phosphorylation and acetylated-Tubulin (Fig. 5E). These results indicate that RGS12 activates MYCBP2 phosphorylation to regulate ciliogenesis.

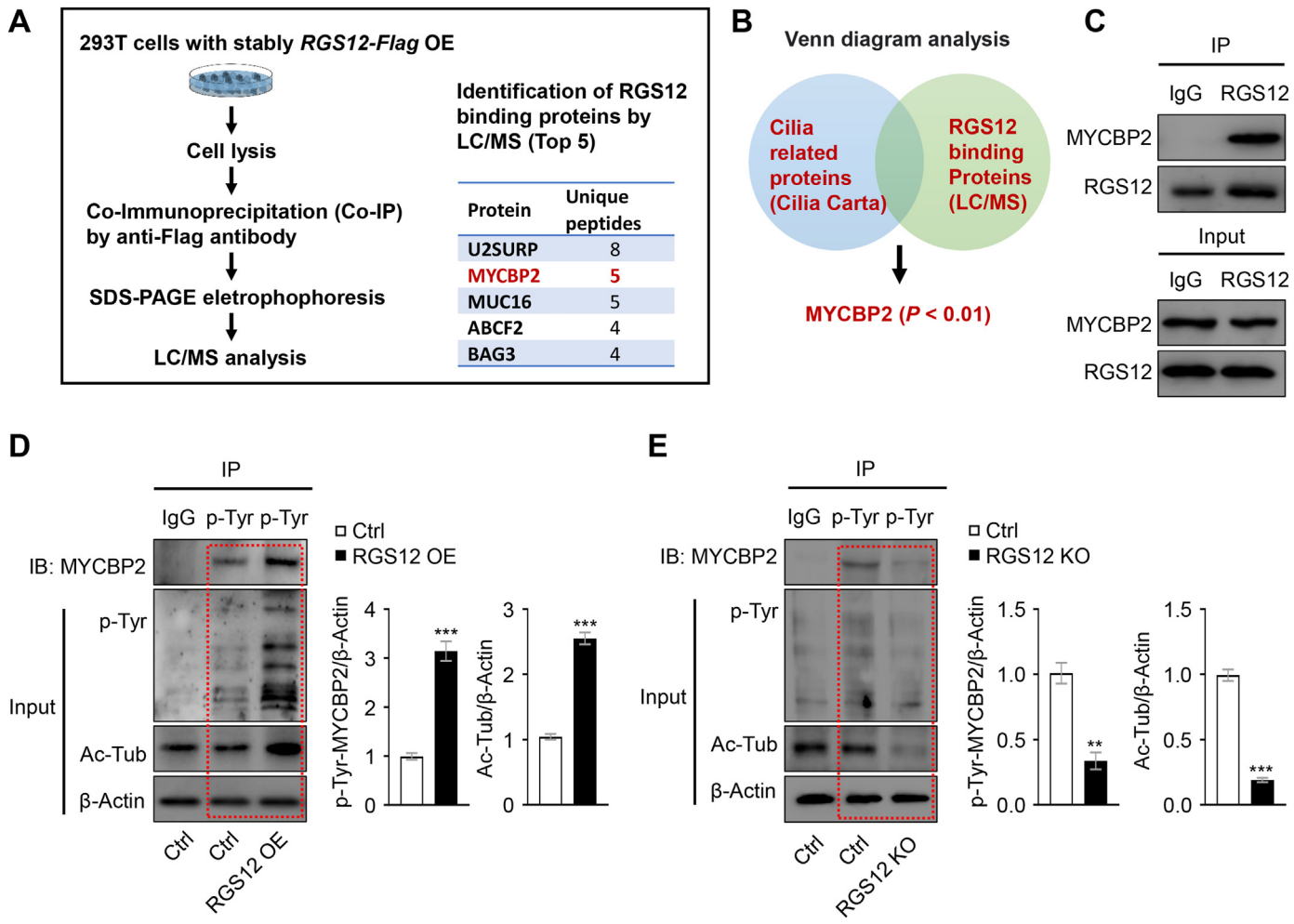

Fig. 5. RGS12 regulates ciliogenesis by association with and activation of MYCBP2. (A) The HEK293T cells were stably transfected with pCMV and pCMV-RGS12-Flag plasmids and the cell homogenates were performed with immunoprecipitation (IP). The immunoprecipitated proteins are fractionated by SDS-PAGE, digested in-gel and analyzed by LC-MS/MS. The top 5 RGS12 binding proteins with unique peptides were shown in the Table. (B) The Venn analysis showed the overlapping proteins between cilia (Cilia Carta database) and RGS12 binding proteins, and MYCBP2 was the most significant protein. (C) MYCBP2 associates with endothelial RGS12. Antibody to RGS12 or a nonspecific isotype-matched IgG was incubated with endothelial cells. Immunoprecipitates were resolved on SDS-PAGE and probed for MYCBP2. (D–E) Phos-Tyr (p-Tyr) interacts with MYCBP2 in endothelial cells (IP assay). The endothelial cell lysates were incubated with anti-Phos-Tyr (p-Tyr) or IgG antibody, and bound protein was examined by western blotting (WB) as indicated. Note that RGS12 OE promoted the level of p-Tyr-MYCBP2 and Ac-Tubulin, whereas the RGS12 KO decreased the level of p-Tyr-MYCBP2 and Ac-Tubulin.

4. Discussion

Increased density of blood vessels in the synovium is a feature of inflammatory arthritis (Paleolog, 2002). The number of synovial blood vessels has been found to correlate with infiltration of inflammatory cells and indices of cartilage damage (Rooney et al., 1988). Interestingly, we found that the expression of RGS12 in endothelial cells is also positively correlated to the number of blood vessels. RGS12 is mainly expressed in endothelial cells by analyzing the Human Atlas. However, under normal circumstances, we found the knockout of RGS12 does not significantly affect the angiogenesis in the synovium. Consistently, one study showed that the RGS12 plays a key role in the cardiovascular system, and the knockout of RGS12 only decreased the myocardial and vascular hypertrophy under the aortic banding model but not the normal conditions (Huang et al., 2016). The possible reason is that RGS12 may be more active in stressful situations such as inflammation or injury. In this study, we should claim that CAG-CreER is a global knockout model rather than a specific vascular RGS12 knockout model, but our in vitro experiments confirmed the important role of RGS12 in endothelial cells. Since arthritis is a disease of various etiologies, we will use a variety of conditional RGS12 knockout mice such as blood vessels, synovium and cartilage in the future to verify its function in treating arthritis.

Primary cilia play important role in inflammation. Inflammatory cytokine interleukin-1 (IL-1) was increased in osteoarthritis, which increases cilia length in chondrocytes and fibroblasts (Wann and Knight, 2012). However, in cells with dysfunction of cilia, the inflammatory response to IL-1 was significantly decreased with the decreased release of nitric oxide (Wann and Knight, 2012). Similarly, we found that loss of RGS12 leads to decreased cilia length and presence in endothelial cells. Without RGS12, the migration and tube formation abilities of endothelial cells were almost abolished under inflammatory conditions. These results suggested that cilia are key organelles for sensing and transmitting inflammatory signals, and endothelial RGS12 controls cilia formation and elongation, which further regualtes the inflammatory responses.

Ubiquitylation is a reversible post-translational modification that is essential for the regulation of immune signaling pathways, in particular NF-κB pathway (Liu et al., 2005). In our previous study, we found that RGS12 promotes ubiquitination and further leads to the degradation of IκB in macrophages (Yuan et al., 2022). In this study, we also found that RGS12 promoted ubiquitination and IκB degradation in endothelial cells (Fig. S5). We further identified that MYCBP2 is the direct Ub ligase regulated by RGS12. In addition, ubiquitination is not only involved in

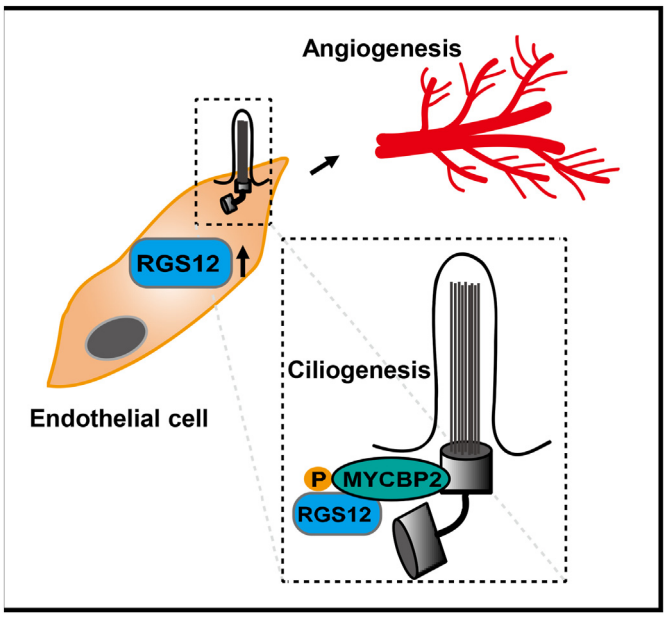

Fig. 6. Schematic for RGS12 regulation of angiogenesis in inflammatory arthritis. In CAIA mice, the increased RGS12 promotes the ciliogenesis and cilia elongation of endothelial cells through association with and activation of MYCBP2. The ciliation of endothelial cells promotes cell migration and angiogenesis and thereby aggravates arthritis.

inflammation but also involved in the regulation of ciliary assembly and disassembly, signal transduction, or ciliogenesis (Hossain and Tsang, 2019). The degradation of cell cytoskeletal proteins or cilia-related proteins affects the function of microtubule-based cilium (Kasahara et al., 2014). The E3 ubiquitin ligase MYCBP2 was found to facilitate the formation and elongation of cilia. Moreover, cilia formation was consistently reduced by 40% in MYCBP2-depleted cells (Quarantotti et al., 2019). Since we found RGS12 is associated with MYCBP2 and ubiquitination, it is suggested that RGS12/MYCBP2 signaling may play an important role in cilia length and presence. Our future study will be focused on the mechanisms of how RGS12 affects the MYCBP2 activation in endothelial cells.

In summary, this study provides the first evidence that endothelial RGS12 promotes the progression of inflammatory arthritis by regulating endothelial cell migration and angiogenesis. Mechanistically, endothelial RGS12 can activate MYCBP2 to promote ciliogenesis and cilia elongation and thereby enhance angiogenesis (Fig. 6). Therefore, this study provides experimental evidence that RGS12 may be a potential anti-angiogenesis drug target for inflammatory arthritis.

Conflict of interest

The authors declare that they have no conflict of interest.

Contributions

S.Y. and G. Y. design the experiments and interpret the results. G.Y. and St.Y. performed and analyzed most of the experiments. S.Y. and G.Y. conceived the project and wrote the manuscript with input from all authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellin.2022.100055.

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