Synovial Fibroblasts Producing Lymphatic Growth Factor, VEGFC, are Decreased During Aging due to cAMP-mediated Reduction of Transcription Factor EGR1

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Introduction. The functions of the synovial lymphatic system (SLS), which maintains joint homeostasis by removing catabolic factors from joints, are impaired in aged joints[1]. Levels of vascular endothelial growth factor C (VEGFC), which is essential for lymphangiogenesis, are decreased in aged synovium, while VEGFC treatment protects against osteoarthritis (OA) and restores SLS function in aged mice[1]. However, the mechanisms mediating decreased VEGFC levels are unknown. By analyzing published sequencing data from OA patients, we found that VEGFC is predominantly expressed by synovial fibroblasts (SF)[1]. Interestingly, our single cell RNA sequencing (scRNA-seq) of synovial cells from 4-m-, 15-m-, and 27-m-old C57Bl/6 male mice revealed an age-dependent decline in the SF population. In the present study, we hypothesize that loss of the transcription factor, Egr1, underlies the decrease in VEGFC-producing SF population during aging, which is mediated by activated cAMP signaling.

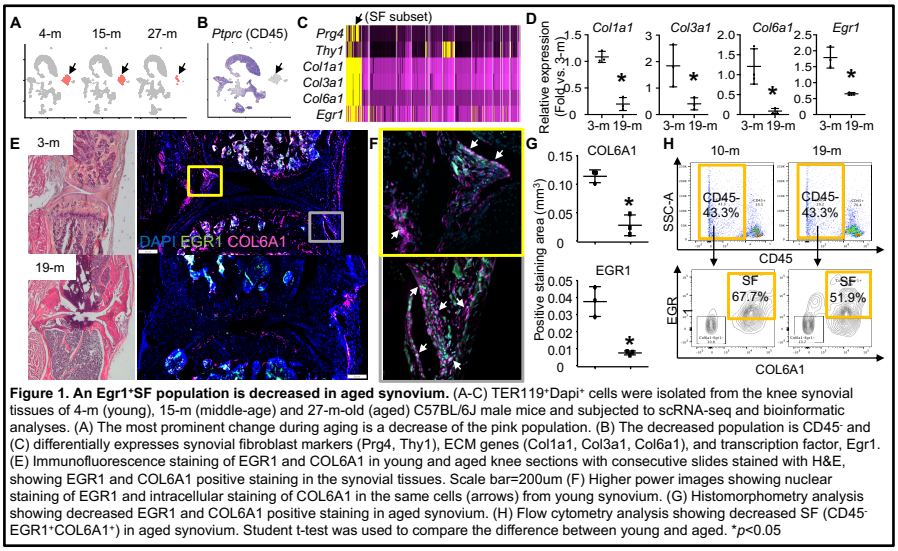

Figure 1. An Egr1⁺SF population is decreased in aged synovium. (A-C) TER119⁺Dapi⁺ cells were isolated from the knee synovial tissues of 4-m (young), 15-m (middle-age) and 27-m-old (aged) C57BL/6J male mice and subjected to scRNA-seq and bioinformatic analyses. (A) The most prominent change during aging is a decrease of the pink population. (B) The decreased population is CD45⁻ and (C) differentially expresses synovial fibroblast markers (Prg4, Thy1), ECM genes (Col1a1, Col3a1, Col6a1), and transcription factor, Egr1. (E) Immunofluorescence staining of EGR1 and COL6A1 in young and aged knee sections with consecutive slides stained with H&E, showing EGR1 and COL6A1 positive staining in the synovial tissues. Scale bar=200um (F) Higher power images showing nuclear staining of EGR1 and intracellular staining of COL6A1 in the same cells (arrows) from young synovium. (G) Histomorphometry analysis showing decreased EGR1 and COL6A1 positive staining in aged synovium. (H) Flow cytometry analysis showing decreased SF (CD45⁻ EGR1⁺COL6A1⁺) in aged synovium. Student t-test was used to compare the difference between young and aged. *p<0.05

Methods. 1. scRNA-seq: 6108, 6210, and 9151 synovial cells from 4-m-, 15-m-, and 27-m-old C57Bl/6 male mice were captured with 10x Genomics followed by 100k reads/cell RNA-seq. Data were analyzed with Seurat4.0 for unsupervised clustering and differentially expressed genes (DEGs). 2: Flow cytometry via a BD cytoperm kit to label intracellular EGR1 and COL6A1 in CD45⁻SFs. 3: Immunofluorescence staining of knee frozen sections from 3-m and 19-m old mice with EGR1 or COL6A1 Abs. Slides were scanned with an Olympus VS120 and histomorphometry was done using Visiopharm software. 4: The Human SW982 synovial cell line was purchased from ATCC and transfected with si-EGR1 RNA (Thermofisher) or treated with the cAMP activator, forskolin (LC Laboratory), for RT-qPCR or Western Blot.

Results. *1. An EGR1⁺SF subset is decreased in aged synovium.* scRNA-seq of the synovial cells revealed a decrease in a CD45⁻ cell population during aging (Fig.1A,B) with differentially expressed SF markers (*Prg4*, *Thy1*), extracellular matrix (ECM) genes (*Col1a1*, *Col3a1*, *Col6a1*), and transcription factor, *Egr1* (Fig.1C), which was validated by RT-qPCR of the synovial tissues from 3-m and 19-m old mice (Fig.1D). Immunostaining of knee sections showed EGR1- and COL6A1-positive cells in the synovial tissues, but not in the meniscus or articular cartilage area (Fig.1E). High power images illustrate nuclear staining of EGR1 and intracellular staining of COL6A1 in the same cells (Fig.1F), corroborating the presence of EGR1⁺ SFs. Histomorphometric analysis of knee sections showed decreased EGR1⁺ and COL6A1⁺ cells in 19-m old mice (Fig.1G). Flow cytometric analysis of synovial cells confirmed decreased CD45⁻EGR1⁺COL6A1⁺ SFs in 19-m old mice (Fig.1H).

2. VEGFC is predominantly produced by EGR1⁺SFs and is decreased after EGR1 knockdown. We previously reported that SLS functions (Fig.2A) and *Vegfc* levels are decreased in aged knees (Fig.2B)[1]. Interestingly, *Vegfc* is differentially expressed by the Egr1⁺SF subset in 4-m joints (Fig.2C). Thus, we speculate that the decrease in *Egr1* causes the reduction in *Vegfc* levels during aging. *EGR1* si-RNA transfection of the human SW982 SF cell line significantly decreased *EGR1* and *VEGFC* gene expression levels (Fig.2D).

3. Activation of the cAMP pathway decreases Egr1 expression. Egr1 is regulated by multiple pathways, depending on the cell type. cAMP activation disrupts serum response factor (SRF) binding to the Egr1 promoter and decreases Egr1 expression[2]. Thus, we treated SFs with forskolin, a cAMP pathway activator via adenylate cyclases (Adcy). We found that forskolin reduced levels of EGR1 (Fig.3A), ECM mRNA (Fig.3B) and *VEGFC* mRNA (Fig.3B). In addition, we found that expression of the positive regulators of the cAMP pathway, *Adcy7* and *Epac1*, increased in the Egr1⁺ SF population during aging in both scRNA-seq data (Fig.3C) and RT-qPCR of the synovial tissues (Fig.3D).

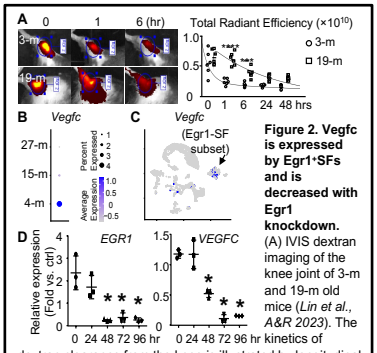

Figure 2. Vegfc is expressed by Egr1⁺SFs and is decreased with Egr1 knockdown. (A) IVIS dextran imaging of the knee joint of 3-m and 19-m old mice (*Lin et al., A&R 2023*). The kinetics of dextran clearance from the knee is illustrated by longitudinal IVIS images at the indicated times following intraarticular injection, with quantification of fluorescence signal intensity (total radiant efficiency). Data for each mouse at each time point were fitted in an exponential decay curve in Matlab showing delayed clearance in 19-m joints. (B) Gene expression of Vegfc is decreased during aging. (C) Vegfc is expressed by Egr1+SFs in a 4-m old joint. (D) VEGFC level is decreased after EGR1 knockdown with siRNA in human SFs. One-way ANOVA was used to assess the difference post transfection compared to ctrl.

Discussion. By combining scRNA-seq analysis and experimental validation, we found that Egr1⁺SFs are the major source of VEGFC in the joint and are decreased during aging (Fig.1&2). However, we do not know if the decrease in VEGFC alone or the reduced Egr1⁺SF population exacerbate age-related OA. Egr1⁺SFs have multiple roles in the joint, including production of extracellular matrix and interaction with immune cells. In addition, our preliminary data (not shown) suggest that Egr1⁺SFs can potentially switch to an inflammatory phenotype upon losing *Egr1* expression. We will investigate this question using the DTA mouse model and conditional deletion of *Vegfc* and *Egr1*. We found that the cAMP pathway may be the specific upstream regulator of Egr1 gene expression in SFs (Fig.3), which is important for the development of novel therapies. EGR1 is an important transcription factor for other cells, including hematopoietic stem cells, and directly modulating EGR1 levels may have off-target effects. Thus, identifying key components, such as Adcy7 and Epac1, that work upstream of EGR1 specifically in SFs may lead to novel therapies targeting Egr1⁺SFs for age-related OA. Specifically, we will explore the effect of ADCY and EPAC1 inhibitors as novel OA therapies.

Significance. Here, we corroborated the scRNA-seq finding of decreased ECM⁺SFs in aged synovium and identified EGR1 as a critical regulator for ECM⁺SFs. In addition, we identified a potential upstream mechanism for the EGR1 decrease in ECM⁺SFs, which may lead to novel therapies for age-related OA.

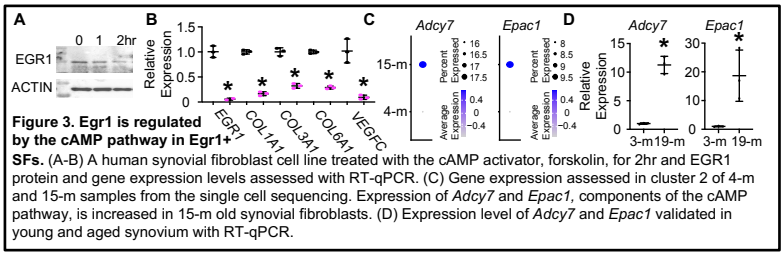

Figure 3. Egr1 is regulated by the cAMP pathway in Egr1⁺ SFs. (A-B) A human synovial fibroblast cell line treated with the cAMP activator, forskolin, for 2hr and EGR1 protein and gene expression levels assessed with RT-qPCR. (C) Gene expression assessed in cluster 2 of 4-m and 15-m samples from the single cell sequencing. Expression of *Adcy7* and *Epac1,* components of the cAMP pathway, is increased in 15-m old synovial fibroblasts. (D) Expression level of *Adcy7* and *Epac1* validated in young and aged synovium with RT-qPCR.

References. 1 *Lin et al., A&R 2023.* 2 *Kimura et al., J Mol Cell Cardiol 2013.*

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