

**Mesenchymal Stem Cell-like properties of orbital fibroblasts in Graves' orbitopathy**

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**Supplementary Figures and Table**

**Methods and Legends**

## Methods

### Immunofluorescence

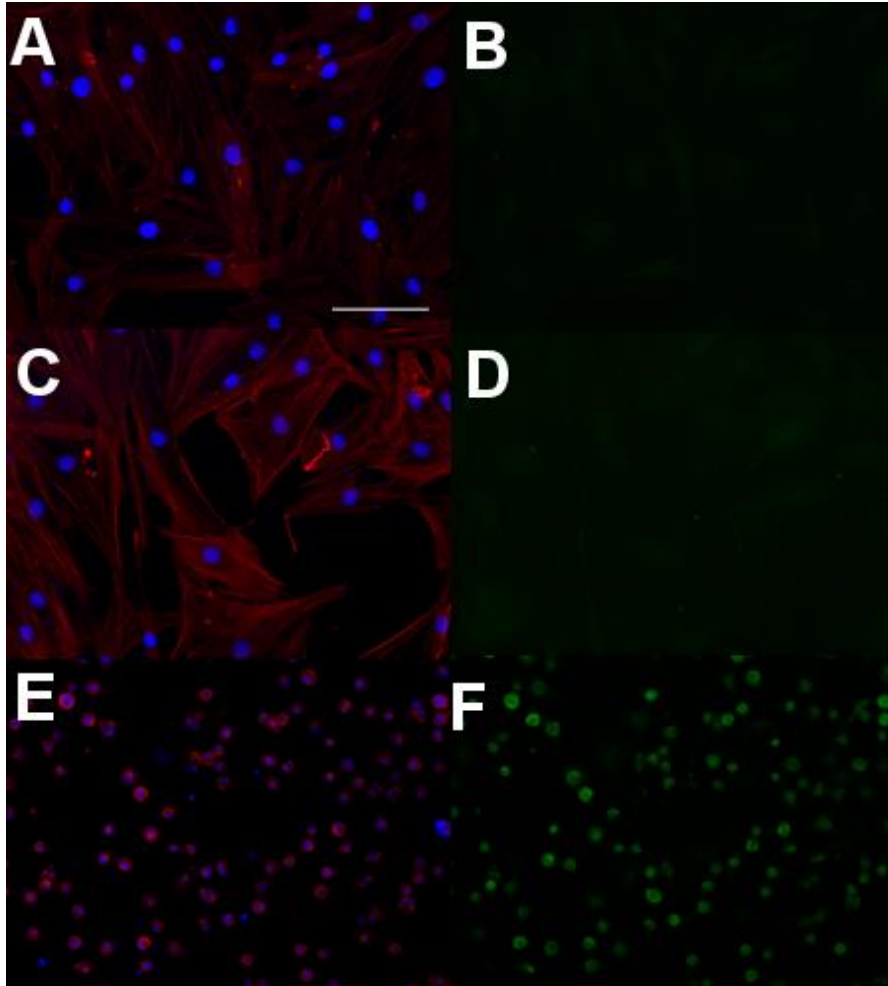
U937 monocytes were transferred to 6-well plates (Fischer Thermoscientific) with glass coverslips, and differentiated into macrophages by adding 1 µg/ml of phorbol 12-myristate 13-acetate (PMA, Promega) to RPMI-1640 medium (Sigma) supplemented with 10% foetal bovine serum (FBS) for three days, then left to recover in RPMI/10%FBS for three days. GO (HO1 line) and control (CO2 line) fibroblasts were detached using enzyme-free cell dissociation buffer (Gibco), plated at  $8 \times 10^3$  cells/cm<sup>2</sup> on glass coverslips as above, and left to attach overnight. All cells were fixed for 7 min with 3.7% formaldehyde in cytobuffer (5 mM KCl, 137 mM NaCl, 4 mM NaHCO<sub>3</sub>, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 1.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 5 mM Pipes, 2 mM EGTA, 5.5 mM glucose, pH 6.1), and permeabilised for 10 min with 0.5% Triton-X100 (Sigma) in cytobuffer. Next, the coverslips were rinsed once with 0.1 M glycine in cytobuffer and incubated for an additional 10 min in glycine/cytobuffer. The samples were then blocked/stained for F-actin by incubation for 20 min with 5 µM Rhodamine-phalloidin (Molecular Probes) in TBS (Tris 20 mM, NaCl 154 mM, pH 8) supplemented with 1% Bovine Serum Albumin (BSA), 1% FBS and 1% donkey serum. The coverslips were then incubated with primary antibodies against CD14 and CD73 (BioLegend, 1:50) for an hour, followed by 3 washes of 10 min with TBS/1%BSA, and a 1hr incubation with Alexa 488-conjugated anti-mouse antibodies (Jackson Labs, 1:100). After final washes, the coverslips were mounted with Fluoroshield mounting medium with DAPI (Abcam). Cells were imaged using Ti-E microscope (Nikon) with CoolSNAP HQ2 camera (Photometrics), using a 20X air objective (20X Plan Fluor ELWD ADM with correction collar).

## **Table Legend**

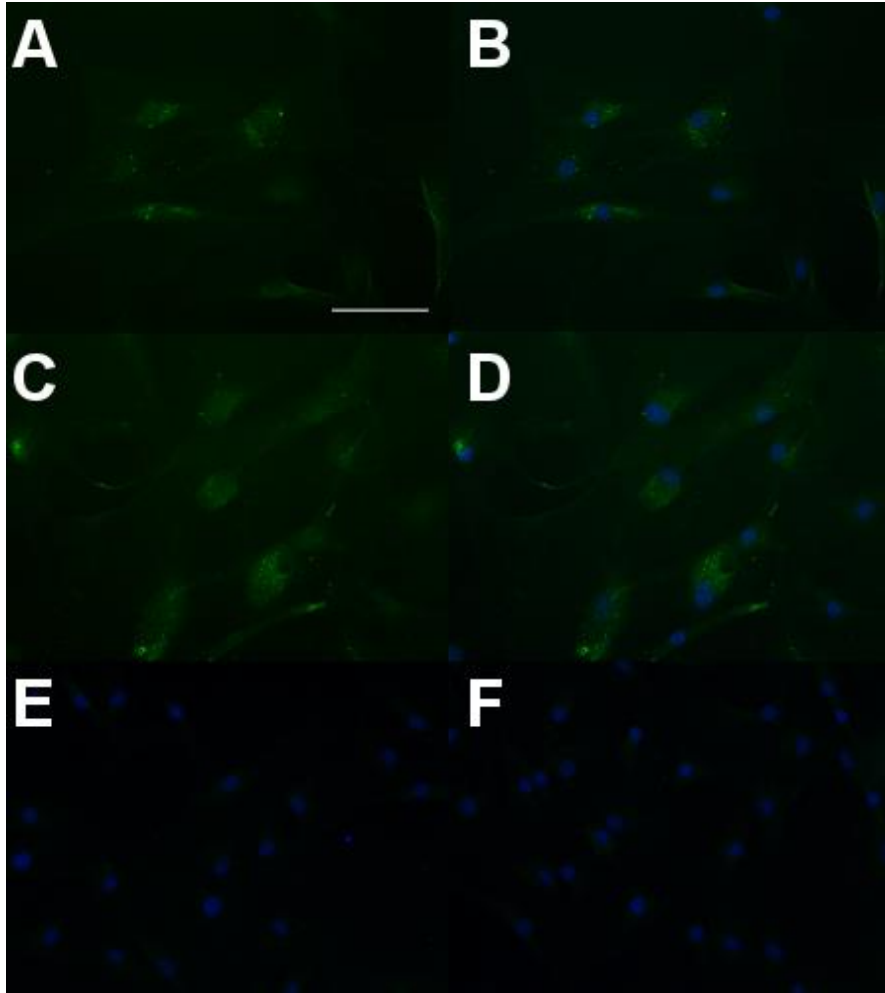
### **Supplementary Table 1. GO fibroblasts express markers of osteogenesis and**

**chondrogenesis.** GO fibroblasts (lines HO1-3) were induced towards osteogenic or chondrogenic differentiation (“differentiated”) or kept in control medium under the same conditions (“undifferentiated”); additionally, cells grown under standard cell culture conditions were tested (“standard”). Expression levels of markers of osteogenesis (BGLAP and SPARC) and chondrogenesis (ACAN, SOX9) were assessed by RT-PCR. Shown is mean the Ct values for 2 independent experiments.

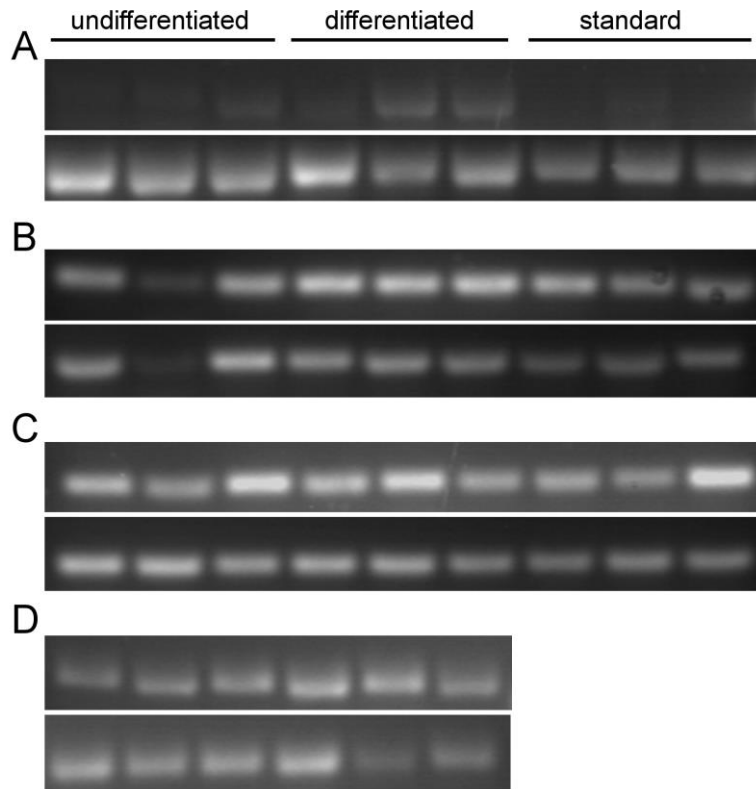
## Figure Legend



**Supplementary Figure S1:** GO and control orbital fibroblasts do not express negative MSC marker CD14. GO and control orbital fibroblasts or macrophages were detached with a trypsin-free solution and immunostained for CD14 as monolayers on glass coverslips following a 24 hr adhesion recovery period. **A, B:** GO cell line HO1; **C, D:** Control cell line CO2; **E, F:** human macrophages used as a positive control. Left panel (**A, C, E**) shows F-actin staining (red) and DAPI (blue). Right panel (**B, D, F**) shows CD14 expression. Scale bar: 100  $\mu\text{m}$



**Supplementary Figure S2:** GO and control orbital fibroblasts express positive MSC marker CD73. GO (**A, B, E**; HO1 cell line) and control (**C, D, F**; CO2 cell line) orbital fibroblasts were detached with a trypsin-free solution and immunostained for CD73 as monolayers on glass coverslips following a 24 hour adhesion recovery period. **A, C:** CD73 staining; **B, D:** CD73 (green) and DAPI (blue); **E, F:** control immunostaining without primary antibody. Scale bar: 100  $\mu\text{m}$ .



**Supplementary Figure S3:** GO fibroblasts express osteogenic and chondrogenic markers.

Agarose gel electrophoresis was performed on end products of RT-PCR (Supplementary Table 1). Two independent repeats were performed, and the figure shows one of the repeats. Samples were loaded as follows: HO1-3 undifferentiated cells, HO1-3 differentiated cells, HO1-3 standard cells, with top lanes showing genes of interest and bottom lane showing their corresponding housekeeping gene controls. A) BGLAP, S3A1; B) SPARC, HPRT1; C) ACAN, HPRT1; D) SOX9, S3A1.