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Supplementary information

Paracrine signalling by cardiac calcitonin controls atrial fibrogenesis and arrhythmia

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SUPPLEMENTARY INFORMATION.

1. SUPPLEMENTARY METHODS.

1.1. Isolation and maintenance of primary human ACMs. Right atrial cardiomyocytes (ACMs) were isolated using a standard enzymatic dispersion technique. Atrial tissue biopsies were collected into ice-cold calcium-free solution containing (mM) NaCl 130, KCl 5.4, MgCl2 3.5., NaH_2PO_4 0.4, HEPES 5, taurine 20 and glucose 10 (pH 7.4 with NaOH). The biopsies were then cut into small (~2-3 mm^2) pieces, rinsed of blood and digested twice (for 12 minutes at 37°C) with type 1 collagenase (2 mg/ml, Worthington) and protease (0.2 mg/ml, Sigma-Aldrich). The following multiple (4-6) digestion steps (for 15 minutes at 37°C) were carried out with protease omitted from the solution. Cells were filtered and pelleted at 300 rpm for 3 minutes and transferred into a solution containing (mM) L-gluthamine 100, KCl 30, HEPES 10, EGTA 1, sodium pyruvate 5, taurine 20, glucose 20, $MgCl_2$ 5, succinic acid 5, creatine 5, Na_2ATP 2 (pH 7.2 with KOH). To measure CT in cell secretome, isolated ACMs were gradually introduced to $CaCl_2$ (1.4 mM final concentration) and incubated for 4-6 hours at room temperature; after centrifugation at 1000 rpm for 3 minutes the ACM secretome was used to measure CT by ELISA (detailed below).

1.2. Sources of other human cells. In addition to the above human primary cells, we also used human TT cells (#CRL-1803, ATCC, USA) obtained from a 77 year old female patient suffering with medullary thyroid carcinoma; human adult dermal fibroblasts (HDFa; #C-013-5C, Gibco, Invitrogen cell culture, USA), HEK293 cells (#CRL-1573, ATCC, USA) and HEK293 stably expressing human CTR (#P30136, Innoprot, USA). All cells were cultured and maintained according to the manufacturer's instruction.

1.3. Western blot. Immunoblotting in human and murine atrial homogenates and in human ACFs was performed using 4%, 6% and 12% gradient acrylamide or 4-12% NuPAGE (ThermoFischer Scientific, USA) gels and the polyvinyl difluoride (PVDF) membranes. The primary antibodies (see details in *Supplementary Table 1*) were raised against CTR (validated previously[1] and in-house, Extended Data

Fig.8b-f), BMP1[2], collagen 1A2[3], α-SMA, total and phosphorylated Erk1/2, filamin A[4], GAPDH, LKB1, calcitonin, αCGRP and pro-CT (validated previously5 and in-house, Extended Data Fig.8g). Antibody for αCGRP/pro-αCGRP (ab189786) was raised against a recombinant full length protein 1-128aa and would recognise the mature peptide, the pro-peptide and all isoforms containing 1-128aa sequence. Human pro-CT/CT was separated using a pre-cast 4-12% gradient NuPAGE Bis-Tris gel (#NP0321, Invitrogen) with NuPAGE MES SDS running buffer (#NP0002, Invitrogen) at constant 140V, while the mouse CT/pro-CT were separated using the in-house made 4-6-16% and 4-12% gradient acrylamide gels and SDS-PAGE running buffer at 90V for 20 minutes (till the samples entered resolving gel) and then at 120V for 1.5 hours. Detection of human pro-CT/CT was performed with an antibody (#130-00025, RayBiotech) raised against 85-116aa sequence corresponding to a mature CT peptide that is present in full length pro-CT (26-141aa). The mouse pro-CT/CT were detected by an antibody (ab8553) raised against 50-141aa sequence present in pro-CT (26-141aa), mature peptide (85-116aa) and katacalcin (121-141aa). Immunodetection of the primary antibodies was performed using horseradish peroxidase-conjugated secondary antibodies (i.e., anti-rabbit IgG (H+L), anti-mouse IgG or anti-goat IgG (H+L). Membranes were developed using a Chemidoc System (BioRad, UK), X-ray film or LICOR odyssey infrared imager and analysed by ImageJ software. In some experiments, immunoblotting was performed in cell lysates or cell-free conditioned medium collected from human ACFs. All normalising proteins and controls were run on the same membrane.

1.4. Accumulation of calcium-rich deposits by fibroblasts was assessed with Alizarin Red S staining (#A5533, Sigma-Aldrich, USA) as previously described[6]**.** Briefly, cells were cultured for 72 hours in the absence or presence of human CT (100 nM) and then fixed with 4% paraformaldehyde for 10 min at 4°C and washed twice with MilliQ water; 200 ml of 1% w/v Alizarin Red S solution was added to each well for 10–15 min, followed by extensive washing with MilliQ water and incubation with 200 ml of de-staining solution (20% methanol, 10% acetic acid in deionized water) for 15–20 min before measuring the absorbance at 405 nm.

1.5. BMP1 Enzyme Activity Assay. BMP1 enzyme activity was measured with a fluorescent assay using fluorogenic substrate, as described previously[7] and according to the manufacturer's instructions (R&D Systems). Briefly, ACFs were cultured in a 96-well plate and maintained until 100% confluent. After 16 hours of starvation in FBS-free medium, cells were pre-treated with vehicle, 100 nM CT or 5 μM of the selective BMP1 inhibitor (3(aminocarbonyl)-b-(3-cyclohexlpropyl)-N-hydroxy-1,2,4-oxadiazole-5-propanamide; #UK383367; R&D Systems) for 24 hours; BMP1 inhibitor was used to confirm BMP1 specificity of the substrate, as the latter can be cleaved by other biologically active enzymes. Conditioned medium with fluorogenic peptide substrate Mca-YVADAPK(Dnp)-OH (#ES007; R&D Systems) in 1:1 ratio was subsequently loaded into a black 96-well plate for the enzymatic activity measurements; fluorescence signal was measured by the fluorescence plate reader (Molecular Devices SpectraMax M2) at the excitation and emission wavelengths of 320 nm and 405 nm respectively. A standard curve was derived from serial dilutions of a standard compound MCA-Pro-Leu-OH (#4018898, Bachem).

1.6. Scar-in-a-jar assay. Collagen-1 accumulation by fibroblasts was assessed using a scar-in-a-jar assay adapted from Chen et al[8]. Briefly, cells were plated at 7000 cells/well overnight in FBM-3 medium (#CC-3131, Lonza) containing 10% FBS and supplement pack (#CC-4525, Lonza). The next morning, cell were starved in serum-free medium for ~1 hour and cultured for the next 72 hours in 0.5% FBS, 100 μM ascorbic acid (#A8960, Sigma-Aldrich), 37.5 mg/ml FICOLL 400 (#F4375, Sigma-Aldrich) containing medium in the presence or absence of 100 nM human CT (#H-2250, Bachem, Switzerland). After 72 hours cells were fixed with ice-cold 100% MeOH for 10 minutes, washed 3 times with PBS and blocked with 3% BSA at room temperature for 1 hour. Following an overnight incubation at 4ºC with primary (anti-Collagen 1 and anti-fibronectin: #C2456 and #F3648 respectively, both from Sigma-Aldrich, USA) antibodies and multiple washing steps, the secondary (A-11001 and A-21245, Life Technologies) antibodies and a fluorescent Hoechst solution (nuclear stain) were added for 1.5 hours. After washing steps (with PBS), imaging of cells using 10x objective

and 9 field per well was performed using the Operetta CLS high content imaging system (Perkin Elmer) and the images were analysed using the Harmony high-content analysis software v3.5 (Perkin Elmer, Revision 105159).

1.7. Real-time quantitative or non-quantitative Polymerase Chain Reaction (PCR). Total RNA was isolated from lysed cells and pulverized or homogenized atrial tissue (using an electric homogenizer Polytron) with an appropriate lysis buffer (TRIzol™ - #15596, Life Technologies, USA), Direct-zol™ (R2072, ZYMO Research, USA), or these from the RNA extraction kits (i.e., Nucleospin RNA II kit - #740955.250, Macherey-Nagel, *mir*Vana™ - #AM1560, ThermoFischer Scientific, USA) following manufacturer's instruction. Complementary DNA (cDNA) was synthesized using high-capacity cDNA reverse transcription kit (#4368814, ThermoFisher Scientific, USA) or iScript™ (#1708841, Bio-Rad, Hercules, CA) supermix according to the manufacturer's protocol. Real-time quantitative PCR (qRT-PCR) was performed with TaqMan fast advanced master mix (#4444557, ThermoFisher Scientific, USA), SYBR Green FastMix (Quanta Biosciences, USA) or Power SYBR Green PCR master mix (#4367659, ThermoFisher Scientific, USA). Gene expression of murine Collagen 1A1 was measured with TaqMan assay, while SYBR Green primers were used to assess transcripts of the collagen 3A1, fibronectin 1 and α-SMA, normalised to hypoxanthine phosphoribosyl transferase 1 (HPRT-1). The primer IDs and sequences are listed in the *Supplementary Table 2.*

In human atrial tissue, ACFs and ACMs, real time qRT-PCR and non-quantitative PCR were used to compare gene expression of CT, αCGRP, CTR, collagen 1A1, collagen 3A1, fibronectin, α-SMA and BMP1 transcripts as previously described[9]. Briefly, total RNA was extracted with miRvana RNA extraction kit (#AM1561, ThermoFisher Scientific, USA) according to the manufacturer's instruction. RT reaction was carried out using the QuantiTect reverse transcription kit (#205313, QIAGEN). For some experiments with a low template concentration or on transfected ACFs, we used cDNA pre-amplification with pre-amp master mix (#4391128, ThermoFisher Scientific, USA) prior quantitative

PCR according to the manufacturer's protocol. Non-quantitative PCR was performed using BioMix Red reaction mix (#Bio-25006, Bioline). Quantitative PCR was carried with TaqMan Fast Advanced Master Mix (#444556, ThermoFisher Scientific, USA) or SsoAdvanced SYBR Green Universal Supermix (#172-5270, Bio-Rad, UK). The primer IDs and sequences are listed in the *Supplementary Table 2.*

Each reaction was performed in duplicates (in human samples) or single replicates (in murine samples) using QuantStudio 7 Flex Real-Time PCR System and ABI 7900HT Detection System (both are from Applied Biosystems). Relative gene expression was calculated using the comparative threshold cycle method (2^ΔCt or 2^ΔΔCt) and normalized to either GAPDH, RS18, U6 snRNA or filamin A, as indicated in each figure; in some cases data were expressed as a fold change (fc) of the respective control group (depicted in the respective figure).

1.8. Histological assessment of atrial fibrosis in mice. Collagen content in murine hearts was assessed using Masson's trichrome staining. Freshly excised hearts were arrested in diastole with 1M/L KCl, fixed in 10% buffered formalin, embedded in paraffin and cut into 6 μm (Montreal cohort) or 7 μm (Houston cohort) sections for Masson's trichrome or sirius red stains (Thermo Fisher Scientific; Waltham, MA, USA) staining. Sections were imaged on a light microscope at 20x, 10x and 4x magnification using Image-Pro 7.0; analysis of the myocardial collagen content was carried out using ImageJ software and expressed as a percentage of the total area. All experiments and analysis were performed blinded.

1.9. Echocardiography of the murine heart. Echocardiographic studies were performed as described previously[10]. Briefly, mice were anesthetized with 1.5-2% isoflurane and kept on a heated platform to maintain the body temperature between 36.5°C and 37.5°C. The VisualSonics VeVo 2100 Imaging System (VisualSonics, Toronto, Canada) equipped with a high-frequency MS505 probe was used to assess cardiac function of all LKB1-based mice; the CTR-KO and respective controls were assessed

by a transthoracic echocardiography using an i13L probe (10-14 MHz) and Vivid 7 Dimension ultrasound system (GE Healthcare Ultrasound, Horten, Norway). LV anterior ($LVAW_d$) and posterior ($LVPW_d$) thickness at the end of diastole and, LV dimension at end cardiac diastole (LVD_d) and systole (LVD_s) were measured by M-mode echocardiography (M-mode) & LV fractional shortening, ejection fraction was obtained by formula available within the Vivid 7 system. LV transmitral filling velocities in early (E) and atrial (A) filling, early filling deceleration time (EDT), deceleration rate (EDR), time interval from mitral valve closure to opening (MV_{CO}) were measured with pulsed wave Doppler (PW). LV fractional shortening (FS) and ejection fraction were obtained by formula available within the Vivid 7 system; LV mass was calculated using a previously described formula[11]. Trans-mitral flow peak velocity in early (E) and atrial (A) filling-phases, early filling deceleration time (EDT), deceleration rate (EDR), time interval from mitral valve closure to opening (MV_{CO}) and pulmonary venous flow peak velocity in systole and diastole (S and D) were measured with pulse-wave Doppler (PW). LV ejection time (LVET) was measured from the beginning to the end of LVOT flow. LV global myocardial performance index (MPI_{global}) was calculated as (MV_{CO} - LVET)/VET X 100%. Enlarged PW was used to sample the flow pattern at the conjunction of LV inflow and outflow to obtain isovolumetric relaxation time (IVRT). Mitral lateral and septal annulus moving velocity in systole (S), early (e') and atrial diastole (a'), and time intervals from the ending of a' to beginning of e' (b), and from the beginning to ending of S (a) were measured by tissue Doppler imaging (TDI). LV regional MPI was calculated by (b-a) / a X 100% for lateral and septum. LVOT dimension was measured by 2D; LVOT flow was traced to obtain stroke volume (SV) and cardiac output (CO). Left atrium dimension at end cardiac systole (LAD_s) was measured by M-mode. The average of 3 consecutive cardiac cycles was used for each measurement, with the operator blinded to treatment assignment.

1. 10. Proteome profiling.

(a) Processing of conditioned medium and de-glycosylation was conducted as previously described[12]. A volume of 0.5 ml of serum-free (i.e. extensively washed before serum starvation) conditioned medium, obtained from cultured human ACFs, was filtered using 3 kDa molecular-mass cut-off (Amicon) and concentrated to 100 µL following the manufacturer's recommendations. Concentrated samples were precipitated using 6:1 (i.e., 600 µl) chilled acetone overnight followed by centrifugation at 14,000 g for 30 minutes. A two-step de-glycosylation protocol was pursued to ensure pan-de-glycosylation. Unlike PNGase-F (acts at a link between the sugar and asparagine residues) the majority of de-glycosylation enzymes act on the sugar residues. Samples were resuspended in 20 µl of deglycosylation buffer (150 mM NaCl, 50 mM sodium acetate pH 6.8 buffer supplemented with 10 mM EDTA) and first incubated with a combination of deglycanation enzymes for GAG-removal (i.e., chondroitinase, heparanase and ketaranase), and an additional 4 enzymes as follows. The Endo-α-N-acetylgalactosaminidase (O-glycosidase from Streptococcus pneumoniae) is used to cleave serine- or threonine-linked unsubstituted Galβ1,3GalNAca; the debranching enzymes - to remove terminal residues α2-3,6,8,9-Neuraminidase (from Arthrobacter ureafaciens), which cleaves all non-reducing terminal branched and unbranched sialic acids; the β1,4-Galactosidase (from Streptococcus pneumoniae) - to release only β1,4- linked, non-reducing terminal galactose from complex carbohydrates and glycoproteins; the β-N-Acetylglucosaminidase (from Streptococcus pneumoniae) - to cleave all non-reducing terminal β-linked N-acetylglucosamine residues from complex carbohydrates and glycoproteins. Removal of sugar monomers facilitates the later cleavage by PNGase-F. After 24-hour treatment, water was evaporated from the extracts and substituted for an equal amount of O18-labeled water containing PNGase-F. This enzyme cleaves all asparagine-linked complex, hybrid, or high mannose oligosaccharides unless α1,3-core fucosylated. Importantly, asparagine must be peptide-bonded at both termini. This step was carried out under constant agitation at 37°C for 24 hours.

(b) In-solution protein digestion and peptide clean-up. De-glycosylated samples were denatured using a final concentration of 6 M urea, 2 M thiourea and reduced by the addition of dithiothreitol (at final concentration 10 mM) followed by 1-hour incubation at 37°C, 240 rpm. Samples were cooled down to room temperature before being alkylated by the addition of 0.5 M iodoacetamide (at final concentration 50 mM) followed by incubation in the dark for 1 hour. Pre-chilled (-20°C) acetone (6x volume) was used to precipitate samples overnight at -20°C. Samples were centrifuged at 16,000 g for 45 minutes at 0°C, supernatants were discarded, protein pellets were dried using a SpeedVac (Thermo Scientific, Savant SPD131DDA) and re-suspended in 0.1 M triethylammonium bicarbonate buffer, pH 8.2. The 0.8 µg of Trypsin/LysC (Promega, V5073) were added after reconstituting the dry enzyme mixture in 50 mM acetic acid. Proteins were digested at 37°C and 240 rpm overnight and the digestion was stopped by acidification of the samples with 10% trifluoroacetic acid (TFA, final conc. 1%). Peptide clean-up was done using a Bravo AssayMAP liquid handling system (Agilent). After conditioning and equilibration of the resin, acidified peptide solutions were loaded onto AssayMAP C18 Cartridges (Agilent, #5190-6532), washed using 1% acetonitrile (ACN), 0.1% TFA (aq) and eluted using 70% acetonitrile and 0.1% TFA (aq). Eluted peptides were vacuum centrifuged to dryness and resuspended in 40 µl of 2% acetonitrile, 0.05% TFA (aq).

(c) Liquid chromatography and tandem mass spectrometry (LC-MS/MS). Cleaned peptides were separated on a nanoflow LC system (Thermo Scientific Dionex UltiMate 3000 RSLCnano). The 2.5 µl of peptide solution were injected onto a trap column (ThermoFisher Scientific, #160454) at a flow rate of 25 µl/min for 3 minutes, using 0.1% formic acid (FA, aq). The following nano LC gradient was then used to separate the peptides at 0.25 µl/min: 0–10 min, 4–10% B; 10–75 min, 10–30% B; 75−80 min, 30−40% B; 80–85 min, 40–99% B; 85–89.8 min, 99% B; 89.8–90 min, 99–4% B; 90–120 min, 4% B; where A = 0.1% FA (aq), and B = 80% ACN, 0.1% FA (aq). The nano column (ThermoFisher Scientific, #ES803A) was kept at 45°C and coupled to an EASY-Spray NG Source (ThermoFisher Scientific). The eluate was sprayed into an Orbitrap Fusion Lumos Tribrid mass spectrometer

(ThermoFisher Scientific) operating in data-dependent Top Speed mode (cycle time 3 seconds). Survey full scan spectra were acquired over the mass-to-charge (m/z) range 350–1500 using Orbitrap detection (resolution of 120,000 at 200 m/z). Dynamic exclusion duration was 60 seconds. Data-dependent MS^2 scans were performed using Quadrupole isolation, collision-induced dissociation activation and Ion Trap detection.

(d) Database search of LC-MS/MS data and data filtering. Proteome Discoverer software (ThermoFisher Scientific, version 2.3.0.523) was used to search raw data files against a hybrid human-bovine database (UniProtKB/Swiss-Prot version of January 2019) using Mascot (Matrix Science, version 2.6.0). A database containing bovine proteins allows for identification of contaminant proteins derived from the bovine serum-containing culture medium initially used to maintain the cells. The mass tolerance was set at 10 ppm for precursor ions and 0.8 Da for fragment ions. Trypsin was used as the protein-digesting enzyme with up to two missed cleavages being allowed. Carbamidomethylation of cysteine was chosen as a static modification. Oxidation of methionine and deamidation of asparagine in the presence of ^{18}O-labelled water were chosen as dynamic modifications. Only high-confidence identifications were accepted, with high confidence being determined by a q-value threshold of 0.01 as determined by the False Discovery Rate (FDR) approach of Proteome Discoverer's Percolator and Protein FDR Validator nodes. Additionally, identified proteins were filtered for a number of unique peptides greater than one. Peptide abundances were determined based on precursor ion intensity.

1.11. Flow Cytometry. Human cultured or freshly isolated ACFs were sorted on a Becton Dickinson (BD) FACS Aria Fusion III sorter using a 100 µm nozzle and FACS Diva software v8. Gating was established based on the live (monitored by DAPI-negative staining) single cells, which were sorted for further analysis into 96 well PCR plates (for SMART-Seq2) or plastic tubes (10x scRNA-seq).

Detection of the surface human CTR (hCTR) protein expression in HEK293 cells (HEK293+hCTR, Extended Data Fig.8f), which prior FC were labelled with an anti-CTR primary antibody (detailed in

Supplementary Table 1) or IgG control and fluorescence-labelled secondary anti-rabbit antibody (Alexa Fluor), was carried out using the same sorter and cells were gated for viable singlets positive for the AF647-positive labelled cells.

1.12. ***SMART-Seq2 scRNA-Seq Data Analysis.*** Raw SMART-Seq2 sequencing data were demultiplexed using Illumina bcl2fastq software (version). Raw sequence reads were quality-checked using FastQC software (Andrews, 2010). Cutadapt (Martin, 2011) software was used to trim poor quality bases (< 20), SMART-Seq2 TSO oligo, poly-Ts and Illumina NextEra adapter sequences from raw reads prior to alignment. Human hg38 reference genome analysis set was obtained from the University of California Santa Cruz (UCSC) ftp site (Kuhn, Haussler and Kent, 2013), indexed as a reference genome and reads were aligned using STAR aligner (Dobin *et al.*, 2013) with the following parameters:

--outFilterMismatchNoverLmax 0.1

--outSAMtype BAM SortedByCoordinate

--outFilterMultimapNmax 50

--outFilterMatchNminOverLread 0.75

--outFilterScoreMinOverLread 0.5

--outSAMattributes All

--outFilterMatchNmin 10

--outFilterMismatchNmax 10

--outReadsUnmapped Fastx

--readFilesCommand zcat

Picard tools (Wysoker, Tibbetts and Fennell, 2013) were used to mark duplicate sequences as an additional QC step. Raw gene expression counts were summarised using feature Counts (Liao, Smyth and Shi, 2013) using a hg38 reference GTF file containing Ensembl transcriptome annotations. Multi QC tool was used to aggregate quality metrics. Sample quality metrics and raw read counts were

imported into R for further processing. R package "scater" (McCarthy *et al.*, 2017) was used for preliminary analysis and computing cell quality control metrics. Wells with low total library size (< 100,000 total reads), low unique read alignment rate (< 50% of total reads) and low library complexity (> 50% of all reads aligned to exonic regions accounted for by the top 50 genes), as well as positive (100 cells) and negative (empty wells) control wells, were filtered out from further analysis. Raw counts were normalised using regularised negative binomial regression approach as implemented in R package 'sctransform' (Hafemeister and Satija, 2019). Top 3000 genes were used as input for dimensionality reduction by PCA. Scree plots were used to determine the amount of variance explained by the principal components and the first 10 principal components were used as input for clustering analysis and dimensionality reduction by UMAP.

A k-Nearest Neighbor (kNN) graph was constructed from cells in the reduced dimension space, using significant principal components as input and other parameters at default. Cells were then clustered using Louvain algorithm for modularity optimisation using kNN graph as input, with resolution parameter set to 0.7. Cell clusters were visualized using UMAP algorithm (McInnes, Healy and Melville, 2018) using the R package "uwot" implementation, with 10 principal components as input as before and n. neighbours =10, spread =1 and min. dist = 0.5. Differential expression analyses were performed using r package 'MAST' (Finak *et al.*, 2015). Benjamini-Hochberb multiple testing correction was used to compute False Discovery Rate (FDR) and genes were considered significantly differentially expressed under 5% FDR.

1.13. Analysis of the droplet-based 10x scRNA-Seq data. For 10x sequence data analysis, raw sequence reads were quality-checked using FastQC software (Andrews, 2010) as before. Human hg38 reference genome analysis set was obtained from the University of California Santa Cruz (UCSC) ftp site (Kuhn, Haussler and Kent, 2013). The Cell Ranger software (version 3.1.0) from 10× Genomics (https://support.10xgenomics.com/single-cell-gene-expression/software/downloads /latest) was used to process, align and summarize unique molecular identifier (UMI) counts against the human hg38

reference genome and corresponding Ensembl gene annotations (obtained using the UCSC Table empty wells were distinguished from barcoded cells using UMI count distributions. 'EmptyDrops' function from Droplet Utils was used to distinguish cells from empty droplets containing only ambient RNA. Furthermore, droplet barcodes for which a high percentage of total UMIs originated from mitochondrial RNAs (>10%) were filtered out, as well as low total UMI count barcodes. Seurat R package was used to normalize expression values for total UMI counts per cell. Highly variable genes were identified by fitting the mean-variance relationship and dimensionality reduction was performed using principal-component analysis (R package irlba). Screen plots were used to determine principal components to use for clustering analyses, as before. As before, cells were then clustered using Louvain algorithm for modularity optimisation using kNN graph as input, with resolution parameter set to 0.3 using the top 7 principal components. Cell clusters were visualized using UMAP algorithm (McInnes, Healy and Melville, 2018) with top 7 principal components as input and n. neighbours =30, spread =1 and min. dist = 0.3.

1.14. Data analysis and sample demultiplexing of the droplet-based 10x scRNA-Seq data. Hashed samples were demultiplexed as follows. Raw UMI counts for each oligonucleotide protein barcode were obtained using cell ranger software, version 3.1.0. Count matrices were first filtered to keep only droplet barcodes from cells passing QC based on mRNA expression profiles, as described above. Filtered hashed feature counts matrix was then used to demultiplex samples as described in (Stoeckius *et al.*, 2018) and implemented in HTO Demux function in r package Seurat. Briefly, counts were normalised using centered log ratio transformation and for each pool, an initial clustering solution was obtained using clara k-mediods clustering with k=1 + number of samples in the pool. For each cluster/hash ID, we then fit a negative binomial distribution and define a positive threshold at 99th percentile of the recovered normalised UMI counts for the hashtag, with cells below this threshold considered negative for the tag. Cell identity is then assigned based on individual hashtag thresholds and multiplets defined as cells positive for multiple tags. Multiplets were filtered out. Cells negative

for all hash tags formed a minor fraction and were also filtered out, following inspection of their mRNA-cluster distributions. Untagged cells correlated with low total mRNA content cells and did not segregate with any particular cluster and thus likely contained unstained/dying cells or free nuclei that have lost their cytoplasm during sample processing. Demultiplexed cells were visualised as tSNE plots from Euclidian distance matrixes. For more information see *Supplementary Methods section 1.17*.

1.15 Gene expression microarrays.

Microarrays were performed on the samples of human ACFs treated with 100 nM CT or vehicle for 72 hours. Briefly, RNA was extracted from thawed cell pellets as described above in section 1.7 and handed over to the Oxford Genomics Centre (Wellcome Trust Centre for Human Genetics, Oxford, UK) for further processing and analysis. RNA was quantified by fluorescent based method and its quality assessment was carried out on the Agilent Bioanalyser; the final normalised RNA quantity was used for further mRNA amplification. mRNA was amplified using the Illumina Total prep kit (Ambion) according to manufacturer's instructions. RNA was labelled and hybridised to Illumina Human HT-12 v4 beadchips which were scanned on an Illumina iScan machine version and data were extracted using the GenomeStudio software version. Raw, probe level summary values were imported into R using beadarray. Probes were background corrected using negative control probes followed by quantile normalization using the neqc command.

1.16. List of software used in the study.

bcl2fastq version 2.20.0.422 - Fastq file conversion from raw bcl format

FastQC Version 0.11.8 Software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) - Raw sequence data quality control software.

STAR Version 2.4.2 - Transcriptome alignment.

Cutadapt Version 1.16 - Sequence data trimming Picard

Subread Version 1.6.2 - feature count summary

Cellranger pipeline version 3.1.0 - 10x raw data processing.

R Version 3.5.3 and 3.4.2 - Language for statistical analysis.

limma 3.34.5

The following packages were used in addition to base installation:

Seurat Bioconductor R Package, Version 3.1.5.9900 - single cell data QC, normalisation, clustering and differential gene expression analysis.

irlba, R Package, Version 2.3.2 - Principal component analysis.

clusterProfiler, Bioconductor R Package, Version 3.6.0 - Gene Ontology enrichment analysis.

ggplot2, R Package, Version 2.2.1 - Plotting package.

DESeq2, Bioconductor R Package, Version 1.20.0 - Differential expression analysis of bulk /pseudobulk RNA sequencing data.

uwot R package Version 0.1.8 - UMAP visualisations

MAST R package, Version 1.8.2 - Single Cell differential gene expression

DropletUtils R package, Version 1.2.2 - data cleaning and cell identification

sctransform R package, version 0.2.1 - data normalisation

FLUOstar Omega software version 5.11, firmware version 1.43 (BMG LABTECH, Germany)

Affymetrix Genechip Command Console software (1.4.1.46)

SoftMax ProSoftware v5.0

ImageJ software (version 1.52a)

Fiji open source software (version 2.0.0-rc-69/1.52i)

DAVID 6.8 web tool

GraphPad Prism (versions v7.05, v6.04, v8.02, or v8.04)

Image-Pro v7.0

PerkinElmer Harmony v3.5 (Revision 105159)

QuantStudio 7 Flex Real-Time PCR System and ABI 7900HT Detection 480 System (both are from Applied Biosystems)

Proteome Discoverer software (ThermoFisher Scientific, v2.3.0.523)

hybrid human-bovine database (UniProtKB/Swiss-Prot version of January 2019)

Mascot (Matrix Science, version 2.6.0)

Cleaned peptides were separated on a nanoflow LC system (Thermo Scientific Dionex UltiMate 3000 RSLCnano)

IOX2 software (version 2.8.0.13 and 2.9.5.28, EMKA technologies, FR)

FACSDiva software v8

RTCA DA v1.0

Gpower v3.1.9.4

1.17. Replication of the results.

Fig.1. Data are pooled from individual donors assessed on the same day in one batch in single replicates (a, d-g), or (b-c, h-i) - in duplicates; all results are reproduced independently twice, or three times (a-c, j, k) in different subjects.

Fig. 2. Data are pooled from individual donor cells assessed on the same day in single replicates except duplicates in (f, i-k). Data in (c) and (l) were pooled from individual donor cells treated on 3 different days assessed on the same day in single replicates. All results were reproduced twice in different donors on different days, or for (a) and (b) - by different method in (g) and (h) respectively.

Fig. 3. Data are pooled from individual donor cells (a-f) treated on the same day and assessed in single replicates on the same day in one batch apart from (g) – assessed over 2-year period. The (e-f) were reproduced twice, except (g) - 3 times in different donors by different experimenters; data in (a) were partially reproduced by other methods (Fig.2a, 2e; Extended Data Fig.2a, 2e). Proteomic analysis was performed in single replicates following validation of reproducibility for (c) in duplicates (see Extended Data Fig.8j).

Fig.4. Data are pooled from individual animals assessed in single replicates on the same day in one batch. Data in (a-c) and (l-m) are pooled from 6-9 sections per animal stained on different days and assessed in one batch. Results in (a-c) were reproduced in another batch of animals by different lab using different method, or by another experimenter using different method (l-m); results in (6i) were repeated on two different days in the same animals. Results in (d-h) and (i-j, l-q) were obtained from individual animals over 2.5-years and 4-month period respectively.

Extended Data Fig.1. Data are pooled from individual donors assessed in single replicates (a, b, f, g-k, m, o-s) or duplicates (c-e, l, n); all results were reproduced independently twice.

Extended Data Fig.2. Data are pooled from individual donor cells assessed in single replicates, except duplicates (a-b, g, i), on the same day in one batch. Results were reproduced twice (a-c, f-h) in different donors. Data in (l-o) are pooled from 6 individual donors in sinus rhythm assessed in 14742 cells (post QC after filtering the initial 18466 total cellular barcodes) on the same day in one batch.

Extended Data Fig.3. The original data in (e) used for the GO analysis were pooled from 6 individual donors assessed in single replicates on the same day in one batch. Data are pooled from individual donors (l), or separate days (m-n) and assessed in single replicates on the same day in one batch apart from (n, single replicates on two different days), or in duplicates in (g, i, k) assessed on the same day. Findings in (a-d, j) were validated by another method (Fig.3a-b, g, Extended Data Fig.6b). All (except e) were reproduced twice in different donors.

Extended Data Fig.4. Data are pooled from 268 single cells isolated from 8 individual donors; scRNA-seq workflow was performed on the same day in one batch.

Extended Data Fig.5. The original data used for the analysis are pooled from 268 single cells isolated from 8 individual donors; the scRNA-seq workflow is carried out on the same day in one batch.

Extended Data Fig.6. Data in (a) are pooled from individual donors assessed in single replicate on the same day; results were reproduced in the same donors twice. Data in (b) are pooled from individual

donors (a few cells in each field as shown in the figure) collected over two-year period, assessed on separate days and validated by 3 independent experimenters.

Extended Data Fig.7. Data are pooled from individual animals assessed in single replicates on the same day and reproduced in two centres in (a, c-d). Results in (m-n) were obtained from individual animals over ~2.5 years.

Extended Data Fig.8. Data in (a-c) are representative images of cells stained on the same day and reproduced three times on three separate days. Data are pooled from individual cultures assessed in duplicates (d), or from technical triplicates (g-h) and technical duplicates (i) analysed on the same day. Blots in (f) were reproduced twice in different donors.

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2. SUPPLEMENTARY TABLES.

Supplementary Table 1. List of the antibodies used in Western blot and immunostaining.

Primary Antibodies	Catalogue #	Manufacturer	Host	Dilution factor	
				Western Blot	Immuno-staining
Calcitonin Receptor	AHP635	Bio-Rad , UK	Rabbit	1/500	1/100
BMP1	ab38953	Abcam, UK	Rabbit	1/1000	N/A
COL1A2 (C-19)	sc-8786	Santa Cruz Biotechnologies, USA	Goat	1/1000	N/A
α-Smooth Muscle	A5228	Sigma, USA	Mouse	1/1000	N/A
p44/42 MAPK (Erk1/2)	9102S	Cell Signaling, USA	Rabbit	1/1000	N/A
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	9101S	Cell Signaling, USA	Rabbit	1/1000	N/A
Filamin A	MAB1680-C	Merck, USA	Mouse	1/1000	1/100
Pro-calcitonin	130-00025-500	RayBiotech , USA	rabbit	1/1000	N/A
GAPDH-HRP-conjugated	G9295	Sigma, USA	N/A	1/1000	N/A
GAPDH	MAB374	Millipore , USA	Mouse	1/1000	N/A
Calcitonin	ab8553	Abcam, UK	Rabbit	1/1000	N/A
αCGRP	ab189786	Abcam, UK	Rabbit	1/1000	N/A
CREB (E306)	ab32515	Abcam, UK	Rabbit	1/1000	N/A
PKA R2 (phospho S96)	ab226754	Abcam, UK	Rabbit	1/1000	N/A
PKA C alpha	AF4268-SP	R&D Systems, UK	Goat	1/1000	N/A
Cyclic AMP	MAB2146-SP	R&D Systems, UK	Mouse	1/1000	N/A
EPAC1 (5D3)	4155S	Cell Signaling, USA	Mouse	1/1000	N/A
EPAC2 (D3P3J)	43239S	Cell Signaling, USA	Rabbit	1/1000	N/A
Collagen 1	C2456	Sigma, USA	Mouse	N/A	1/100
Fibronectin	F3648	Sigma, USA	Rabbit	1/1000	1/100

Supplementary Table 2. List of primer sequences and TaqMan assay IDs used in qRT-PCR and PCR.

Target transcript	Forward Primer	Reverse Primer	Target transcript	TaqMan Assay ID
Mouse			**Mouse**	
Collagen 3A1	AGCTTTGTGCAAAGTGGAACC	GGTGGCTGCATCCCAATTCA	mouse collagen 1A1	Mm00801666_g1
Fibronectin 1	GAAGGCAATGGACGCATCAC	CCTTCTTGCTCCACGTGTCT	**Human**	
α-SMA	AAGACAGCTATGTGGGGGATG	GGCCACACGAAGCTCGTTA	human CTR	Hs00156229_m1
HPRT-1	CAGTCCCAGCGTCGTGATTA	TGGCCTCCCATCTCCTTCAT	human CT	Hs01100741_m1
LKB1	CCGCCTGCTCCATATTTCCA	TCGCTGTCTTTGGGTTGGTT	human BMP1	Hs00241807_m1
Calca	GAGGGCTCTAGCTTGGACAG	AAGGTGTGAAACTTGTTGAGGT	human collagen 1A1	Hs00164004_m1
CGRP	CTCTCAGCACGATATGGGTCC	GCAAGAGATGTTTTTCCTGGTCG	human collagen 3A1	Hs00943809_m1
Human			human GAPDH	Hs02786624_g1
CT_CALCA	CACGCAGGACTTCAACAAGTTT	TGCTCCAACCCCAATTGC	human RS18	Hs01584357_g1
αCGRP_CALCA	TCATTGCCCAGAAGAGAGCC	CAAAGTTGTTCTTCACCACACC	human α-SMA	Hs05005341_m1
GAPDH	ACCCAGAAGACTGTGGATGG	TTCTAGACGGCAGGTCAGGT	human filamin A	Hs00924645_m1
CTR1a	GATTCCCACTGATCCACCCA	CTTCTCCTTGGTATGCGGGT	human U6 snRNA	001973
CTR1b	ACCCTGCGGCTGACATCT	CCAAGGCTCCTGAAAAACACG		

Supplementary Table 3. List of the Biolegend Total-seq A hashing antibodies used in a droplet-based 10x single-cell RNA-sequencing of cultured human ACFs.

Antibody	Catalog #	Manufacturer	Dilution
TotalSeq™-A0251 anti-human Hashtag 1	394601	BioLegend	1 µg/ml
TotalSeq™-A0252 anti-human Hashtag 2	394603	BioLegend	1 µg/ml
TotalSeq™-A0253 anti-human Hashtag 3	394605	BioLegend	1 µg/ml
TotalSeq™-A0254 anti-human Hashtag 4	394607	BioLegend	1 µg/ml
TotalSeq™-A0257 anti-human Hashtag 7	394613	BioLegend	1 µg/ml
TotalSeq™-A0258 anti-human Hashtag 8	394615	BioLegend	1 µg/ml
TotalSeq™-A0259 anti-human Hashtag 9	394617	BioLegend	1 µg/ml
TotalSeq™-A0263 anti-human Hashtag 13	394625	BioLegend	1 µg/ml
TotalSeq™-A0264 anti-human Hashtag 14	394627	BioLegend	1 µg/ml
TotalSeq™-A0260 anti-human Hashtag 10	394619	BioLegend	1 µg/ml
TotalSeq™-A0255 anti-human Hashtag 5	394609	BioLegend	1 µg/ml
TotalSeq™-A0256 anti-human Hashtag 6	394611	BioLegend	1 µg/ml