et al., 2014; Iyer et al., 2015; Bao et al., 2017; Guadix et al., 2017;

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Fig. 2. Characterisation of hPSC-epi heterogeneity by scRNA-seq. (A) Principal component analysis of the gene expression in hPSC-epi cells, showing some of the main gene influences on PC2. (B) Distribution of expression of TCF21, WT1 and BNC1 in all epicardial cells (232). The numbers of cells for which no expression is detected are 105, 154 and 44, respectively (represented by the thick line at the bottom of the graph). Boxes represent the inter-quartile range (IQR) between quartile 1 (Q1=25%) and quartile 3 (Q3=75%); whiskers represent Q1-1.5×IQR and Q3+1.5×IQR. (C) Principal component analysis of the epicardial cells, coloured by the expression of TCF21, WT1 and BNC1 (see key above), showing the strong alignment with PC2. The lower-right panel presents the overlap of TC21 (in red) and BNC1 (in turquoise) showing that their expression is exclusive. (D) WT1 and BNC1 detected by immunofluorescence in hPSC-epi. (E) BNC1 distribution in human epicardium at 8 weeks pc. Arrows point towards high-expressing cells, filled arrowheads towards low-expressing cells and empty arrowheads to negative cells. (F) WT1 and BNC1 detected by immunofluorescence in epicardial explant cultures from embryonic human heart at 8 weeks pc. The pink arrowheads point toward a single BNC1-positive cell, the blue ones towards double-negative ones. The other cells displayed on the images are double positive. Scale bars: 30 µm (D); 9 µm (E); 20 µm (F).

was expressed at a level 13 times higher in TCF21^{high} than in BNC1^{high} cells. (Table 1). Immunofluorescence confirmed that the distribution of the protein THY1 was indeed negatively correlated with WT1 in our system (Fig. 5A) and to WT1 and BNC1 in primary human foetal epicardial explants (Fig. 5B,C). As TH had not been reported before in the epicardium, we validate expression on cryosections of human embryonic hearts at 8 w pc. Immunofluorescence confirmed a heterogeneous expressi of

THY1 in the human developing epicardium (Fig. 5D). We used an anti-THY1 antibody to magnetically separate the two epicardial populations from constitutive GFP-expressing (GFP

GFP-negative (GFP

We measured the expression of WT1 and TCF21. WT1 was downregulated 4-fold (Fig. 8B) whereas TCF21 was upregulated 6-fold (Fig. 8C) when hPSC-epi was differentiated under a low level

deleterious secreted factors. However, the situation with hPSC-epi subpopulations may not be that simple. For example, fibronectin has been reported to have positive or negative effects depending on the context (Wang et al., 2013). Moreover, loss of TCF21 leads to abnormal EPDCs (Braitsch et al., 2012). Furthermore, our bioinformatics analysis suggested that the angiogenic potential of the epicardium resides in the TCF21^{high} population. So instead of using a pure BNC1^{high} hPSC-epi population for cell therapy following myocardial infarction, it may be preferable to engineer a BNC1^{high}-enriched population but with attenuated rather than absent CF potential.

Our work has shown that using the hPSC-epi in association with next-generation single cell sequencingB2Aes-0.4 (317.6 (25 (pre)-274.5 (pr)22.3 be)-ee)18 (y (BN8ion)-60)2 (in)he2ine8PSC281(ch(

in a 1:1 ratio, supplemented with Glutamax-I, chemically defined lipid concentrate (Life Technologies), transferrin (15 µg/ml, Roche Diagnostics), insulin (7 µg/ml, Roche Diagnostics), monothioglycerol (450 µM, Sigma-Aldrich) and polyvinyl alcohol (PVA, 1 mg/ml, Sigma-Aldrich)] on gelatin-coated plates. The cells were first differentiated into early mesoderm with FGF2 (20 ng/ml), LY294002 (10 µM, Sigma-Aldrich) and BMP4 (10 ng/ml, R&D Systems) for 36 h. Then, they were treated with FGF2 (20 ng/ml) and BMP4 (50 ng/ml) for 3.5 days to generate

-10.1 (ydn)se 5-16.3 (h)-13.6 (e)-9.9due2md6.5 (a)-9.9 (r)-18.2 (llbidueR-13.6 (0)-267 4 (l)223.6 (e)a13.6 (s)-150.3 (e)-f-

MATERIALS AND METHODS

Tissue culture

hPSC-derived cells

hPSCs (H9 line, Wicell) were maintained as previously described (Iyer et al., 2015) and tested every 2 months for mycoplasma contamination. hPSC differentiation was performed in CDM-PVA [Iscove's modified Dulbecco's medium (Gibco) plus Ham's F12 NUT-MIX (Gibco) medium

Fig. 8. BNC1 function in developing epicardial cells. (A) hPSC-epi developed from TET-inducible knockdown hPSC showed more than 90% reduction in *BNC1* RNA under the TET condition (Aa) and 98% reduction at the protein level by western blot (Ab) as also visualised by immunofluorescence (Ac) (n=5). (B,C) These cells showed more than 75% reduction of *WT1* RNA (B) and a 5-fold increase in *TCF21* RNA (C). (D,E) When BNC1 is silenced during its development, the hPSC-ep2h 6 (a)cmc56 (0-15.4 1 Tf 0515 (-e)27. (n)13.9 ()13.9 (soSe2iS)2Td [().)415.3 (n)13.9-15pw_3 1 Tf 3.16 (e)11.7ar)2Td [().).9 (bl)-8.8 ()().).9 (bn)27.6-e)277-8

preparation kit and Index Kit (Illumina) were used for cDNA tagmentation and indexing. Library size and quality were checked using an Agilent High Sensitivity DNA chip with Agilent Bioanalyser (Agilent Technologies). The pooled libraries of 96 cells were sequenced at the Babraham Institute sequencing facility on an Illumina HiSeq2500 at 100 bp per read. We used one lane per plate, resulting in 250,000 to 5,800,000 reads per sample.

The quality of the raw data were assessed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) for common issues including low quality of base calling, presence of adaptors among the sequenced reads or any other over-represented sequences, and abnormal per base nucleotide percentage. FASTQ files were mapped to the *Homo sapiens* genome GRCh38 using HISAT2 (Kim et al., 2015). We removed the 22

samples (over 384) for which either most of the reads (above 97%) were mapped to the ERCC spike-in, probably representing empty wells, without cells, or for which less than 80% of reads were in genes, or for which less than 2% genes were detected. This represented 2-13 samples per 96-well plate. Of the remaining 362 cells, 130 were from the lateral plate mesoderm stage (hPSC-LM) and the 232 others from hPSC-epi. The data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE122827.

Preliminary analysis using PCA showed that a few cells were isolated, far from most of their grouped siblings. Those cells had fewer reads than others and a low gene count. We therefore removed 36 cells with fewer than 500,000 reads, and expressing fewer than 7000 genes. The expression of genes was quantified using SeqMonk RNA-Seq pipeline (https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). Raw read counts aligned with all exons were summed for each gene.

Bulk sequencing

Total RNA was extracted from cultures using the RNeasy mini from Qiagen. DNA contamination was removed from the samples using the DNA-free DNA Removal Kit from Ambion (Thermo Fisher Scientific). cDNA alignedan37952 (a25213343378 (128267.1 (55.32 1 377he)-367.1 (D5.3.3.276.-1)e)-28)-256.7 .1

transfection mix at 37°C overnight before washing in CDM-BSA II media the next day approximately 18 h post-transfection. After 2 days, 1 μg ml^{-1} of puromycin was added to the CDM-BSA II culture media. Individual hPSC clones were picked and expanded in culture in CDM-BSA II following 7-10 days of puromycin selection.

Genotyping siKD hPSC clones

Clones from gene targeting were screened by genomic PCR to verify site-specific targeting, determine whether allele targeting was heterozygous or homozygous, and check for off-target integrations of the targeting plasmid. (See Table S6 for PCR primers and thermocycling conditions and Fig. S6 for PCR results.) All PCRs were performed using 100 ng of genomic DNA as template in a 25 μl reaction volume using LongAmp Taq DNA Polymerase (NEB) according to the manufacturer's instructions, including 2.5% volume dimethyl sulphoxide (DMSO). DNA was extracted using the genomic DNA extraction kit from Sigma-Aldrich according to the manufacturer's instructions.

Inducible BNC1 knockdown

One homozygous-targeted clone for each vector transfection was selected for subsequent differentiation into hPSC-epi with or without the addition of 1 μg/ml tetracycline (Sigma-Aldrich) to culture media with the aim of mediating BNC1 knockdown. hPSC-epi was successfully differentiated from each clone in the presence and absence of tetracycline. qPCR analysis indicated that clone 1Ei had a very pronounced reduction in BNC1

Author contributions

Conceptualization: L.G., S.S.; Methodology: L.G., S.A.M., V.M., A.D., S.A., W.G.B., B.G., N.G.L.N.; Software: D.S., A.D.; Validation: L.G., S.A.M., M.A.M.; Formal analysis: L.G., S.A.M., D.S., A.D., S.A., N.G.L.N.; Investigation: L.G., S.A.M., M.A.M.; Resources: L.G., P.R.R., S.S.; Data curation: L.G., D.S., A.D., S.A., N.G.L.N.; Writing - original draft: L.G., N.G.L.N., S.S.; Writing - review & editing: L.G., S.A.M., P.R.R., B.G., N.G.L.N., S.S.; Visualization: L.G., S.A.M., D.S., A.D., N.G.L.N.; Supervision: L.G., W.G.B., P.R.R., B.G., S.S.; Project administration: S.S.; Funding acquisition: L.G., P.R.R., S.S.

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Data availability

RNA sequencing data have been deposited in Gene Expression Omnibus under accessions numbers GSE122827 and GSE122714.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.174441.supplemental

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