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ARTICLE

Proteomic characterisation reveals active Wnt-signalling by human multipotent stromal cells as a key regulator of beta cell survival and proliferation

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Abstract

Aims/hypothesis Novel strategies to stimulate the expansion of beta cell mass in situ are warranted for diabetes therapy. The aim of this study was to elucidate the secretome of human bone marrow (BM)-derived multipotent stromal cells (MSCs) with documented islet regenerative paracrine function. We hypothesised that regenerative MSCs will secrete a unique combination of protein factors that augment islet regeneration.

Methods Human BM-derived MSCs were examined for glucose-lowering capacity after transplantation into streptozotocin-treated NOD/severe combined immunodeficiency (SCID) mice and segregated into samples with regenerative (MSC^{R}) vs nonregenerative (MSC^{NR}) capacity. Secreted proteins associated with islet regenerative function were identified using stable isotope labelling with amino acids in cell culture (SILAC)-based quantitative proteomics. To functionally validate the importance of active Wnt signalling, we stimulated the Wnt-signalling pathway in MSC^{NR} samples during ex vivo expansion using glycogen synthase kinase 3 (GSK3) inhibition (CHIR99201), and the conditioned culture media (CM) generated was tested for the capacity to support cultured human islet cell survival and proliferation in vitro.

Results MSC^{R} showed increased secretion of proteins associated with cell growth, matrix remodelling, immunosuppressive and proangiogenic properties. In contrast, MSC^{NR} uniquely secreted proteins known to promote inflammation and negatively regulate angiogenesis. Most notably, MSC^{R} maintained Wnt signalling via Wnt5A/B (~2.5-fold increase) autocrine activity during ex vivo culture, while MSC^{NR} repressed Wnt signalling via Dickkopf-related protein (DKK)1 (~2.5-fold increase) and DKK3 secretion. Inhibition of GSK3 activity in MSC^{NR} samples increased the accumulation of nuclear β-catenin and generated CM that augmented beta cell survival (13% increases) and proliferation when exposed to cultured human islets.

Conclusions/interpretation Maintenance of active Wnt signalling within human MSCs promotes the secretion of matricellular and proangiogenic proteins that formulate a niche for islet regeneration.

Keywords Angiogenesis · Diabetes · Inflammation · Islets · Mass spectrometry · Multipotent stromal cells · Proteomics · Wnt signalling

Electronic supplementary material The online version of this article (doi:10.1007/s00125-017-4355-7) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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Abbreviations

7AAD	7-Aminoacetinomycin D
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
BM	Bone marrow
BMP	Bone morphogenetic protein
CM	Conditioned culture media

DKK	Dickkopf-related protein
EBM	Endothelial basal media
ECM	Extracellular matrix
EdU	5-Ethynyl-2′-deoxyuridine
EGM	Endothelial growth medium
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FLT1/4	Vascular endothelial growth factor receptor 1/4
Flz3	FluoZin-3
FZD	Frizzled
GSK3	Glycogen synthase kinase 3
HMVEC	Human microvascular endothelial cells
IIDP	Integrated Islet Distribution Program
KDR	Vascular endothelial growth factor receptor 2
LFQ	Label-free quantification
MMP	Matrix metalloproteinase
MSC	Multipotent stromal cell
MSC^{NR}	Nonregenerative MSC
MSC^{R}	Regenerative MSC
PDGF	Platelet-derived growth factor
SCID	Severe combined immunodeficiency
SFRP1	Secreted frizzled-related protein 1
SILAC	Stable isotope labelling with amino acids in cell culture
STZ	Streptozotocin
TIMP	Metalloproteinase inhibitor
VEGF	Vascular endothelial growth factor receptor
WISP2	Wnt-inducible signalling pathway protein 2

Introduction

In 2013, WHO reported that over 347 million people worldwide lived with diabetes, and estimated that this number will increase to 552 million by 2030, making diabetes a worldwide epidemic [1]. The Edmonton Protocol provided proof-of-concept that islet transplantation can temporarily reduce insulin dependence in individuals with type 1 diabetes [2]. Although islet replacement is an attractive strategy to combat diabetes, a critical shortage of donor islets and eventual rejection by continuing autoimmunity prevent the widespread application of this approach [3]. Bone marrow (BM)-derived stem cell transplantation has also been reported to promote endogenous islet regeneration in preclinical models, and represents a promising alternative strategy to combat diabetes [4].

Human multipotent stromal cells (MSCs) have become a focal point in recent clinical trials for tissue repair [5]. MSCs are readily available from autologous or allogenic donors, they are efficiently expanded in culture and they home to damaged tissues to initiate innate repair mechanisms [6]. MSCs exert their therapeutic effects primarily through the secretion of trophic signals within damaged organs, or impact tissue repair from distant sites by secretion of regenerative effectors into the circulation within exosomes or microvesicles [7]. Exosomes harvested from MSCs have been shown to contain proangiogenic proteins and promote the healing of ischaemic tissue [8, 9]. MSCs also secrete a wide variety of immunomodulatory molecules that dampen autoimmunity via modulation of immune cell functions [10, 11]. Currently, the mechanisms underlying the regenerative and immunomodulatory effects of MSCs remain poorly understood, and better understanding of MSC protein secretion is required to harness the true regenerative capacity of MSCs.

In the context of diabetes, transplanted human BM-derived MSCs have been shown to promote repair of pancreatic islets and renal glomeruli in NOD/severe combined immunodeficiency (SCID) mice after beta cell ablation [6]. Although MSC conversion into insulin-expressing beta-like cells has been demonstrated after stable induction of PDX1 [12], minimally manipulated MSCs did not adopt a beta cell phenotype after transplantation in vivo. In contrast, transplanted MSCs initiated endogenous islet recovery via paracrine stimulation [13]. In a series of publications, we have shown that human BM-derived MSCs stimulated the emergence of small, recipient-derived islet-like structures associated with the ductal epithelial niche within 7 days of injection into streptozotocin (STZ)-treated hyperglycaemic NOD/SCID mice [14, 15]. Unfortunately, MSC samples showed donor-dependent variability in the capacity to improve glycaemia and prolonged expansion ex vivo reduced islet regenerative prowess [14]. Thus, detailed proteomic analyses of the islet regenerative MSC secretome remain the key to understanding which protein signals promote islet regeneration in situ.

We compared the secretory protein profile of human BM-derived MSCs with or without islet regenerative capacity by performing global proteomic analysis of conditioned culture media (CM) after stable isotope labelling with amino acids in cell culture (SILAC) [16]. We hypothesised that islet regenerative MSCs will secrete a combination of unique protein factors that augment islet regeneration. In order to confirm our findings we also examined the effects of exogenous stimulation of Wnt signalling in MSCs for the survival and proliferation of human islet-derived beta cells in vitro. To our knowledge, this is the first proteomic study linking the human MSC secretome profile with beta cell regenerative function and it highlights the importance of active Wnt signalling in the maintenance of MSC-induced islet regeneration.

Methods

MSC^{R} reduced hyperglycaemia after transplantation in vivo Human BM was obtained from healthy donors after informed consent at the London Health Sciences Centre (London, ON, Canada). All studies were approved by the human ethics committee at Western University (2015–003).

The hyperglycaemia-lowering capacity of six MSC samples was assessed after tail vein injection of 500,000 cells into STZ-treated NOD/SCID mice as previously described [14]. Blood glucose concentrations were monitored weekly for 42 days and samples were segregated into regenerative (MSC^{R}) or nonregenerative (MSC^{NR}) based on the ability to reduce blood glucose compared with PBS-injected control mice.

MSC culture and SILAC labelling MSCs were expanded in AmnioMax media (Invitrogen Carlsbad, CA, USA). At passage 3 MSCs were switched to custom AmnioMax that contained no L-arginine or L-lysine. Heavy [$^{13}C_6$ $^{15}N_4$] L-arginine and [$^{13}C_6$ $^{15}N_2$] L-lysine were added into SILAC media at 87.8 mg/l and 52.2 mg/l, respectively (Silantes Germany). Excess unlabelled L-proline was added to prevent conversion of heavy arginine into heavy proline [17]. MSCs were grown in SILAC media for 9 days to achieve >95% label incorporation.

Generation of labelled CM and proteomic workflows MSCs were re-plated in AmnioMax without supplement for 24 h to collect proteins secreted by MSCs. CM was collected in biological and technical duplicate, filtered and concentrated using 3 kDa cut-off filter units (Millipore, Bedford, MA, USA). Total protein (150 μg) was subjected to 1D SDS-PAGE fractionation using 12% gels and quantified using the bicinchoninic acid assay (BCA). All MS data was collected using a QExactive (ThermoFisher Scientific, Waltham, MA, USA). Full MS parameters are outlined in the electronic supplementary material (ESM) Table 1. Data analysis was performed using PeaksStudio 7.5 and MaxQuant 1.5.2.8. See ESM Methods for complete details.

HMVEC tubule forming assay To assess CM influence on endothelial cell function in vitro, 120,000 human microvascular endothelial cells (HMVECs) were cultured on growth factor-reduced Geltrex (Life Technologies, Carlsbad, CA, USA) in endothelial basal media (EBM-2; Lonza, NJ, USA) conditioned by MSC^{R} and MSC^{NR} for 24 h. As a positive control, HMVECs were also grown in Geltrex bathed in complete endothelial growth medium (EGM-2 = EBM-2 + 5% FBS + IGF, basic fibroblast growth factor [FGF], EGF, vascular endothelial growth factor [VEGF]). Tube formation was quantified by counting the number of complete tubule branches in four fields of view using ImageJ software (NIH, Bethesda, MD, USA).

qPCR for β-catenin Real-time quantitative PCR (qPCR) was performed using SYBR Green along with the human WNT Signalling Pathway PCR Array, which contained 84 genes related to Wnt signal transduction (Qiagen, Hilden, NW, USA) using mRNA isolated from MSC^{R} vs MSC^{NR}. See ESM Methods for complete details.

Confocal microscopy and flow cytometry for total β-catenin MSCs were cultured in six-well plates on glass cover slips and treated with glycogen synthase kinase 3 (GSK3) inhibitor CHIR99021 (EMD Millipore, Billerica, MA, USA) and stained for nuclear β-catenin. Flow cytometry was used to quantify total cytosolic β-catenin levels after treatment with CHIR99201. See ESM Methods for complete details.

Human islet culture with MSC CM Human islets from five donors were obtained from the Integrated Islet Distribution Program (IIDP). Upon arrival, 200 islet equivalents were plated in RPMI media without serum (Invitrogen). MSC CM was collected after 24 h from MSCs treated with DMSO (basal CM) or with 10 μmol/l of CHIR99021 (GSK3i CM). CM was concentrated using 3 kDa molecular mass cut-off filters, and ~1.0 μg/μl total protein was added to human islet culture for 1, 3 or 7 days (ESM Fig. 1). After islet harvest and dissociation, beta cell content was estimated using FluoZin-3 (Flz3) (ThermoFisher Scientific) and apoptosis was quantified using 7AAD and Annexin-V. To detect islet cell proliferation, 500 nmol/l of 5-ethynyl-2′-deoxyuridine (EdU) was added to islet culture 24 h prior to harvest. Islets were fixed and permeabilised using 10% formalin and saponin buffer, stained for insulin using a PE-conjugated insulin antibody (R&D Systems, Minneapolis, MN, USA), and nuclear EdU incorporation was detected using the Click-It flow cytometry assay (Life Technologies). Flow cytometry data were analysed using FloJo software (Treestar, Ashland, OR, USA).

Statistical analysis Statistical analysis was performed using GraphPad Prism version 6.01 (GraphPad, La Jolla, CA, USA) by ANOVA with Tukey's post hoc test or by multiple *t* tests. Data are expressed as means (SEM).

Results

Regenerative capacity of human MSCs was donor specific Hyperglycaemic (15–25 mmol/l), STZ-treated (35 mg/kg per day, days 1–5) NOD/SCID mice were i.v. injected on day 10 with MSCs (5×10^5) [14, 18], and blood glucose levels were monitored for 42 days. Compared with PBS-injected control mice, MSC samples ($n = 3$) that showed significant reduction in systemic blood glucose from days 17–42 were termed MSC^{R} and MSC samples ($n = 3$) that did not show blood glucose reduction (>25 mmol/l) were termed MSC^{NR} (Fig. 1a). There was a significant reduction in blood glucose AUC for MSC^{R} vs MSC^{NR} samples (Fig. 1b). Out of a total of 18 MSC lines tested, six (~33%) were characterised as MSC^{R}, four (~22%) demonstrated intermediate regenerative capacity and eight (~44%) were characterised as MSC^{NR}. MSC lines with intermediate regenerative capacity were not used in this study. All MSC^{R} and MSC^{NR} lines possessed multipotent

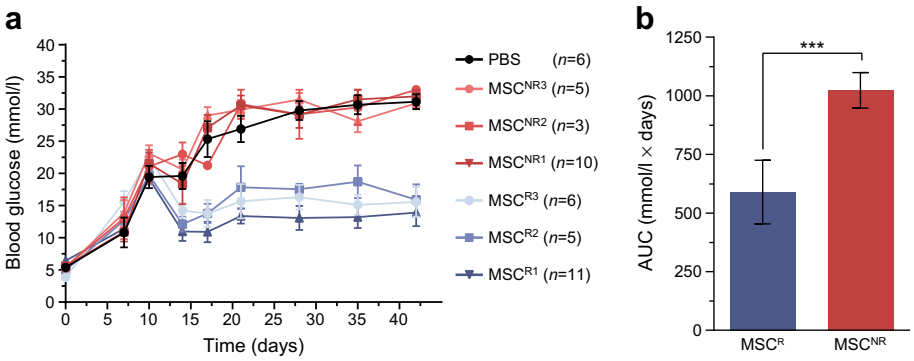

Fig. 1 Functional characterisation of islet MSC^{R} and MSC^{NR}. (**a**) MSCs from six donors were injected into STZ-treated (35 mg/kg per day, days 1–5) NOD/SCID mice on day 10, and blood glucose was monitored weekly until 42 days to segregate MSC^{R} (blue) vs MSC^{NR} (red) samples compared with PBS-injected control mice (black). Blood glucose concentrations were lower after injection of MSC^{R} compared with MSC^{NR} samples. (**b**) Blood glucose AUC was significantly reduced with MSC^{R} (n = 3) (blue) vs MSC^{NR} (n = 3) (red) samples. (**c**) Donor characteristics were similar between MSC^{NR} and MSC^{R} samples. Data are presented as mean ± SEM. ***$p < 0.001$

c

Cell line	Age	Sex	Weight (kg)	BMI (kg/m^2)
MSC^{NR1}	40	M	103.3	31.5
MSC^{NR2}	34	M	77.0	27.3
MSC^{NR3}	29	M	102.0	29.5
MSC^{R1}	32	M	71.3	24.4
MSC^{R2}	32	M	71.2	22.5
MSC^{R3}	54	M	103.0	33.6

differentiation potential into adipogenic and osteogenic lineages in vitro [14, 15]. Cell surface phenotype showed that both MSC^{R} and MSC^{NR} expressed the stromal markers CD90 and CD105 (>95%) without expression of the pan-leucocyte marker CD45 (data not shown). MSC donor information, including age, sex, weight and BMI, is shown in Fig. 1c.

MSC^{R} exclusively secreted proteins associated with active Wnt signalling We have previously shown that MSC^{R} demonstrated increased transcription of matrix metalloproteases, EGF-family ligands and downstream products of Wnt signalling [15]. Here, we sought to confirm and identify secreted protein targets that contribute to islet regeneration by comparing the secretome of MSC^{R} and MSC^{NR} samples using global MS-based proteomics coupled with SILAC. CM was harvested from each MSC line (24 h) and analysed in biological and technical duplicates. The total number of proteins detected for each MSC sample is shown in Fig. 2a. Proteins from each MSC line were combined based on regenerative capacity and complete lists of secreted proteins detected exclusively in CM from MSC^{R} or MSC^{NR}, or proteins common to both groups, were generated (ESM Tables 2–4). Of 4665 total proteins detected (false discovery rate 1%), 3023 (~65%) were produced by both MSC^{R} and MSC^{NR}, 850 (~18%) were produced by MSC^{R} exclusively, while 792 (~17%) were produced by MSC^{NR} exclusively (Fig. 2b). Gene ontology using cellular component analysis revealed that MSC^{R} CM contained qualitatively more proteins with extracellular localisation (21% vs 14%), while MSC^{NR} CM contained more membrane-bound proteins (23% vs 19%) (Fig. 2c). Moving forward, protein lists were filtered to include only extracellular (secreted) and membrane-bound proteins, and were further analysed for known biological functions and signalling family association.

MSC^{R} demonstrated increased representation of secreted proteins associated with angiogenesis and activation of the Wnt-signalling pathway, while MSC^{NR} showed increased secretion of proteins associated with EGF, FGF and Notch signalling pathways (Fig. 2d). MSC^{R} exclusively secreted several proteins associated with cell survival and growth, such as FGF7 and bone morphogenetic protein (BMP)4. Proangiogenic factors vascular endothelial growth factor receptor 2 (KDR), vascular endothelial growth factor receptor 4 (FLT4) and regulators of angiogenesis A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)13 and 18 were also unique to the MSC^{R} secretome [19]. Notably, MSC^{R} secreted potentiators of Wnt signalling (spondins), ligand activators of Wnt signalling (WNT5B) and downstream products of Wnt signalling that modify the extracellular matrix (ECM) (Wnt-inducible signalling pathway protein 2 [WISP2/CCN5]) (Table 1) [20–22]. In contrast, proteins exclusively detected in MSC^{NR} CM included positive and negative regulators of angiogenesis (FGF receptor (FGFR)4, FLT1) [19, 23], and an abundance of proinflammatory cytokines (IL-6, IL-8) and C-X-C motif chemokines (CXCL2, 3, 5) (Table 2) [24]. Collectively, these findings suggested MSC^{R} actively secreted cell growth supportive factors with reduced secretion of common proinflammatory signals, and only MSC^{R} showed production of multiple effectors associated with active Wnt signalling.

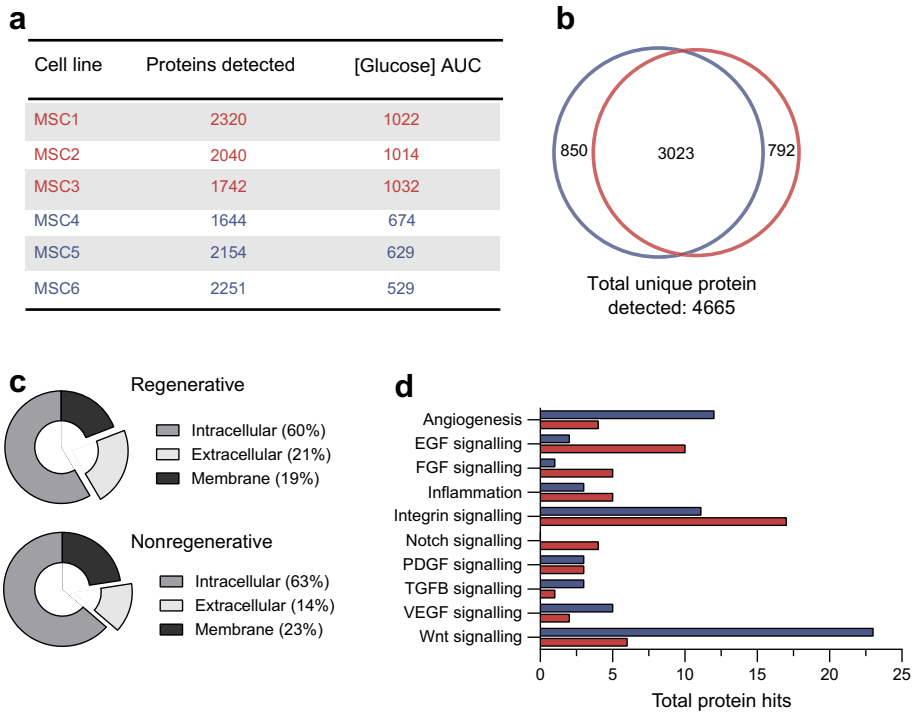

Fig. 2 Qualitative analyses of proteins exclusively secreted by MSC^{R} or MSC^{NR}. (**a**) Total proteins identified for MSC^{R} (blue) and MSC^{NR} (red) lines using SILAC. (**b**) Venn diagram showing that out of the 4665 total proteins detected, 850 were unique to MSC^{R} (blue) and 792 were unique to MSC^{NR} (red). (**c**) MSC^{R} demonstrated a higher proportion of proteins with extracellular (secreted) localisation compared with MSC^{NR}. (**d**) MSC^{R} secreted more proteins associated with the activation of Wnt signalling and angiogenesis. MSC^{NR} secreted more proteins associated with EGF, FGF and Notch signalling

Quantification of Wnt, matrix remodelling and proangiogenic proteins To quantify proteins secreted by both MSC^{R} and MSC^{NR}, quantitative proteomics was performed using label-free quantification [16, 25] to identify proteins differentially secreted into serum-containing media. Lists were filtered to include only secreted and membrane-bound proteins, and 1038 common proteins were quantified to generate lists of differentially expressed proteins (ESM Table 5). We did not observe any significant difference in the total number of proteins quantified between samples (Fig. 3a), and 468 proteins were differentially expressed between MSC^{R} vs MSC^{NR} samples ($p < 0.05$, Fig. 3b). Upregulated proteins in MSC^{R} CM included matrix remodelling proteins (matrix metalloproteinase (MMP)1, MMP3, ADAMs and BMPs) [26, 27], effectors of Wnt signalling (WNT5A, secreted frizzled-related protein 1 [SFRP1]) [21], additional targets implicated in angiogenesis (angiogenin [ANG], angiopoietin [ANGPT]1, ANGPTL2, TGFB1, TGFB2, platelet-derived growth factor

Table 1 Exclusively detected proteins in CM from MSC^{R} involved in angiogenesis, inflammation, matrix remodelling and Wnt signalling

Gene symbol	Protein name	Biological function
ADAMTS13	A disintegrin and metalloproteinase with thrombospondin motifs 13	Matrix metalloproteinase
ADAMTS18	A disintegrin and metalloproteinase with thrombospondin motifs 18	Matrix metalloproteinase
EGF	Epidermal growth factor	Regulator of cell growth, proliferation and differentiation
FGF7	Fibroblast growth factor 7	Tissue repair, mitogenic cell survival
FLT4	Vascular endothelial growth factor receptor 3	Development of vascular networks via secretion of VEFGA/C
IGF2	Insulin-like growth factor 2	Growth promoting hormone
KIT	Mast/stem cell growth factor receptor Kit	Stem cell maintenance, migration, survival and proliferation
KDR	Vascular endothelial growth factor receptor 2	Regulation of angiogenesis and vascular development
MMP10	Stromelysin-2	Matrix metalloproteinase
MMP12	Macrophage metalloelastase	Matrix metalloproteinase
MMP3	Stromelysin-1	Matrix metalloproteinase
PDGFD	Platelet-derived growth factor D	Regulation of cell proliferation, migration and chemotaxis
SPON2	Spondin-2	Activator of Wnt signalling
TIMP4	Metalloproteinase inhibitor 4	Matrix metalloproteinase inhibitor
WISP2	Wnt1-inducible-signalling pathway protein 2	CCN5, regulator of cell growth
Wnt5b	Protein Wnt5b	Activator of Wnt signalling

Table 2 Exclusively detected proteins in CM from MSC^{NR} involved in angiogenesis, inflammation, matrix remodelling and Wnt signalling

Gene symbol	Protein name	Biological function
CXCL2	C-X-C motif chemokine 2	Inflammatory response
CXCL3	C-X-C motif chemokine 3	Inflammatory response
CXCL5	C-X-C motif chemokine 5	Inflammatory response
GDF15	Growth/differentiation factor 15	Regulation of inflammation and apoptosis
FLT1	Vascular endothelial growth factor receptor 1	Negative regulator of VEGFA signalling
FGFR4	Fibroblast growth factor receptor 4	Regulation of angiogenesis and vascular development
FZD1	Frizzled-1	Wnt signalling receptor
FZD2	Frizzled-2	Wnt signalling receptor
FZD7	Frizzled-7	Wnt signalling receptor
IL-1B	Interleukin-1 beta	Inflammatory response
IL-6	Interleukin-6	Inflammatory response
IL-8	Interleukin-8	Inflammatory response
MMP11	Stromelysin-3	Matrix metalloproteinase
NOV	Protein NOV homologue	CCN3, promoter of cell differentiation
TGFBR2	TGF-beta receptor type-2	Regulator of cell growth, proliferation and differentiation

receptor [PDGFR]A, B) [23, 28] and chemokines involved in cell recruitment (CXCL12) (Fig. 3c) [28, 29]. Thus, the activation of Wnt and proangiogenic signalling again correlated with islet regenerative potency. Conversely, the negative regulator of Wnt signalling Dickkopf-related protein (DKK)1 was increased ~2.5-fold in MSC^{NR} CM, and negative regulators of angiogenesis (ADAMTSs, thrombospondin [THBS]1 and THBS2) and inhibitors of matrix remodelling proteins (metalloproteinase inhibitor [TIMP]3) were increased in MSC^{NR} CM [30]. HMVECs were cultured on growth factor-reduced Geltrex to assess the angiogenic potential of MSC CM in vitro (Fig. 3e). A significant increase in the tubule formation was observed when HMVECs were cultured using CM from MSC^{R} compared with MSC^{NR}. Representative photomicrographs of tube formation after 24 h are shown in ESM Fig. 2. Finally, we identified that pro-differentiation proteins associated with Notch signalling were upregulated in MSC^{NR} (NOTCH2, JAG-1) [31], suggesting that inhibited Wnt and angiogenic signalling and activated Notch signalling was associated with diminished islet regenerative capacity.

MSC^{R} increased expression of Wnt pathway mRNA To confirm the activation of Wnt signalling in MSC^{R}, we assessed mRNA expression of 84 genes related to Wnt pathway signal transduction, using three MSC^{R} and three MSC^{NR} lines performed in duplicate. Transcripts with differential expression greater than twofold between MSC^{R} vs MSC^{NR} revealed 18 significantly changing genes (ESM Fig. 3a,b), normalised to the geometric mean of three housekeeping genes: *ACTB*, *B2M* and *HPRT1*, that did not show significant variation across samples. Notably, all differentially expressed mRNAs (18 genes) were upregulated in MSC^{R}, and no significantly changing genes were downregulated. Upregulation of *WNT5A/B* in MSC^{R} were confirmed at the mRNA level. Other upregulated mRNA in MSC^{R} included transcription factors (*FOSL1*, *JUN*) and receptors associated with Wnt signalling (*FZD5*) [20, 32]. Taken together, upregulation of Wnt signalling at the mRNA and protein level strongly suggests active Wnt signalling during expansion was a unique and conserved characteristic that correlated with the reduction of hyperglycaemia after transplantation of MSC^{R} in vivo.

MSCs show activation of Wnt signalling via accumulation of nuclear β-catenin Canonical Wnt signalling converges on the actions of β-catenin, a transcription factor that increases the expression of downstream effectors of Wnt signals [33]. We mimicked Wnt signalling in MSC^{NR} and MSC^{R} using a small molecule (CHIR99201) inhibitor of GSK3. GSK3 actively phosphorylates β-catenin, which marks it for ubiquitination and degradation. Therefore, inhibition of GSK3 leads to stabilisation and accumulation of free β-catenin [20]. MSC samples showed maximal increases in total β-catenin using 5–10 μmol/l CHIR99201 for 24–48 h. We then used confocal microscopy to visualise the accumulation of nuclear β-catenin (Fig. 4). Compared with DMSO control cells (Fig. 4a), MSC^{NR} and MSC^{R} stimulated with 10 μmol/l CHIR99201 showed increased nuclear β-catenin localisation (Fig. 4b, c). Next, MSC^{NR} and MSC^{R} samples were analysed in quadruplicate to quantify total β-catenin levels by flow cytometry (Fig. 4d). Representative dot plots are shown in

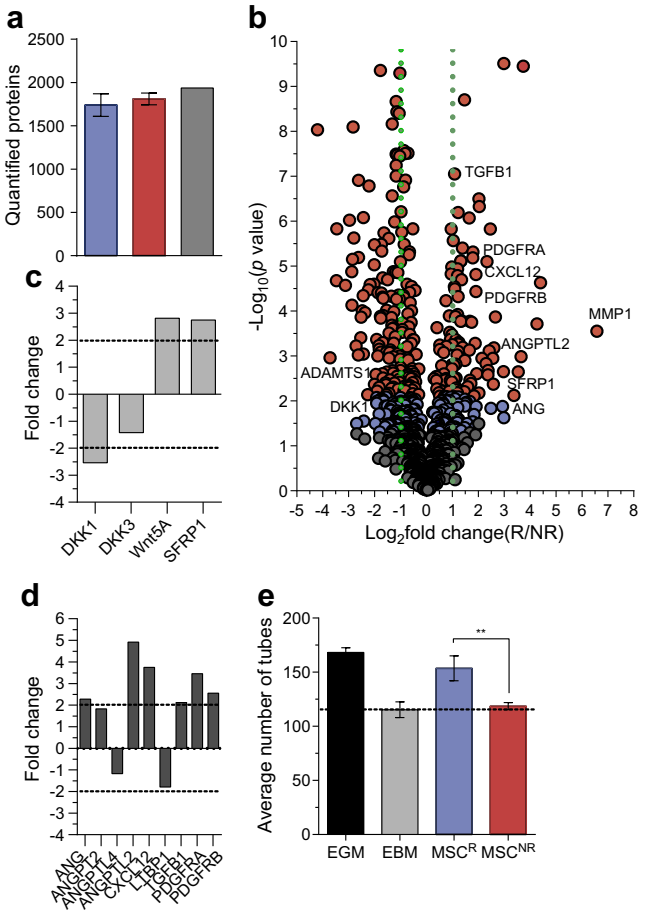

Fig. 3 Quantitative analyses of proteins secreted by both MSC^{R} and MSC^{NR}. (**a**) Total number of proteins quantified within all three MSC^{R} (blue), MSC^{NR} (red) samples and total number of quantified proteins (grey). (**b**) Representative volcano plot of differentially expressed secreted proteins. A change greater than twofold is represented outside the green boundaries. Red, $p < 0.01$; blue, $p < 0.05$; grey, $p > 0.05$. (**c**) MSC^{R} showed increased secretion of Wnt activators (Wnt5A), while MSC^{NR} showed increased secretion of Wnt inhibitors (DKK1). (**d**) MSC^{R} demonstrated increased secretion of proangiogenic proteins, TGFB1 and SDF-1 (CXCL12). (**e**) Spontaneous tubule formation of HMVECs on growth factor-reduced Geltrex was increased when cultured using CM generated from MSC^{R} compared with MSC^{NR}. Data are presented as mean ± SD. $^{**}p < 0.01$

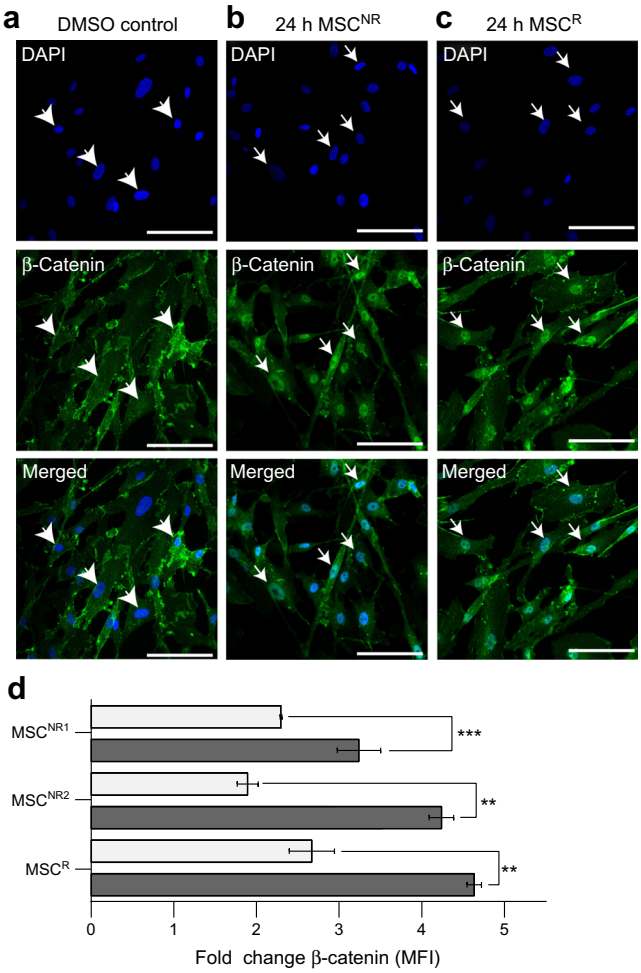

Fig. 4 MSC treatment with GSK3 inhibitor induced nuclear β-catenin localisation. Representative photomicrographs showing MSC^{R} and MSC^{NR} stained for β-catenin (green) and DAPI (blue) after treatment with (**a**) DMSO vehicle, (**b**) CHIR99201 (24 h) MSC^{NR}, (**c**) CHIR99201 (24 h) MSC^{R}. White arrows indicate examined nuclei with nuclear staining and arrowheads indicate examined nuclei without nuclear staining. Scale bars, 50 μm. (**d**) Intracellular β-catenin was increased in both MSC^{R} and MSC^{NR} treated with CHIR99201 (grey bars) or DMSO control (white bars). Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$

ESM Fig. 4. Compared with DMSO control cells, both MSC subtypes treated with CHIR99201 showed significantly increased total β-catenin. Thus, inhibition of GSK3 with CHIR99201 mimicked activated Wnt signalling in MSC^{R} and MSC^{NR}.

GSK3 inhibition in MSC^{NR} generates CM that improves human beta cell survival in vitro To assess whether MSC^{R} CM could improve beta cell survival in vitro, we cultured primary human islet preparations for up to 7 days in CM from MSC^{R} and MSC^{NR}, and performed multiparametric flow cytometry to analyse beta cell survival and proliferation. Human islets were obtained through the IIDP. Compared with MSC^{NR} CM, human islets grown in MSC^{R} CM showed increased total number of beta cells (Fig. 5a) and an increased frequency of live beta cells (Fig. 5b) after 7 days of culture. To further assess the influence of active Wnt signalling on islet regenerative paracrine function, we also assessed whether CM generated by MSC^{NR} treated with a GSK3 inhibitor (CHIR99201) during expansion could promote human beta cell survival or proliferation in cultured human islets. CM recovered from MSC^{NR} cultured with vehicle control (DMSO) only (basal CM) was compared with CM recovered from MSC^{NR} treated with 10 μmol/l CHIR99201 (GSK3i CM) (Fig. 5c). Five independent human islet samples were cultured as indicated in ESM Fig. 1. At each time point, human islets were harvested, dissociated with trypsin, and stained using Flz3 to estimate

beta cell frequency, 7AAD to determine cell viability and Annexin-V to determine apoptosis rates by multiparametric flow cytometry. Representative dot plots are shown in ESM Fig. 5. Compared with basal CM, human islets grown in CM generated by GSK3-inhibited MSC^{NR} showed increased total cell numbers (Fig. 5d), beta cell numbers (Fig. 5e) and the proportion of live (7AAD−) beta cells at 7 days culture (Fig. 5f). However, no significant changes in the frequency of apoptotic beta cells were observed (Fig. 5g).

To determine whether increased beta cell number was augmented by increased beta cell proliferation in vitro, we measured EdU incorporation in insulin + beta cells. At each time point, human islets were harvested, permeabilised, stained for intracellular insulin and proliferation was detected by EdU incorporation using the Click-It system. Representative dot plots showing EdU+ cell detection for each condition are shown in ESM Fig. 6. Compared with basal CM, islet exposure to CM generated by GSK3-inhibited MSC^{NR} increased the overall frequency of proliferating cells (Fig. 6a) and proportion of total proliferating beta cells (Fig. 6b). Finally, islet donor variability was documented by donor information including age, sex, BMI and average blood glucose levels (Fig. 6c). Collectively, these data suggest that CM generated from MSC^{NR} treated with CHIR99201 promoted beta cell survival and induced beta cell proliferation in vitro.

Discussion

We used comprehensive SILAC-based proteomic analyses to identify MSC-secreted factors that correlated with the capacity to lower circulating blood glucose after transplantation into STZ-treated NOD/SCID mice. MSC^{R}, demonstrated glucose-lowering capacity after transplantation, secreted proteins associated with cell growth, matrix remodelling, immunosuppressive and proangiogenic properties. In contrast, MSC^{NR}, which lacked islet regenerative functions, secreted proteins involved in the initiation of inflammation and the negative regulation of angiogenesis. Notably, MSC^{R} consistently demonstrated mRNA and protein expression associated with active Wnt signalling, a novel signature that correlated with islet regenerative capacity. Inhibition of GSK3 activity with CHIR99201 mimicked Wnt signalling in MSC^{NR} and resulted in the generation of CM that supported beta cell

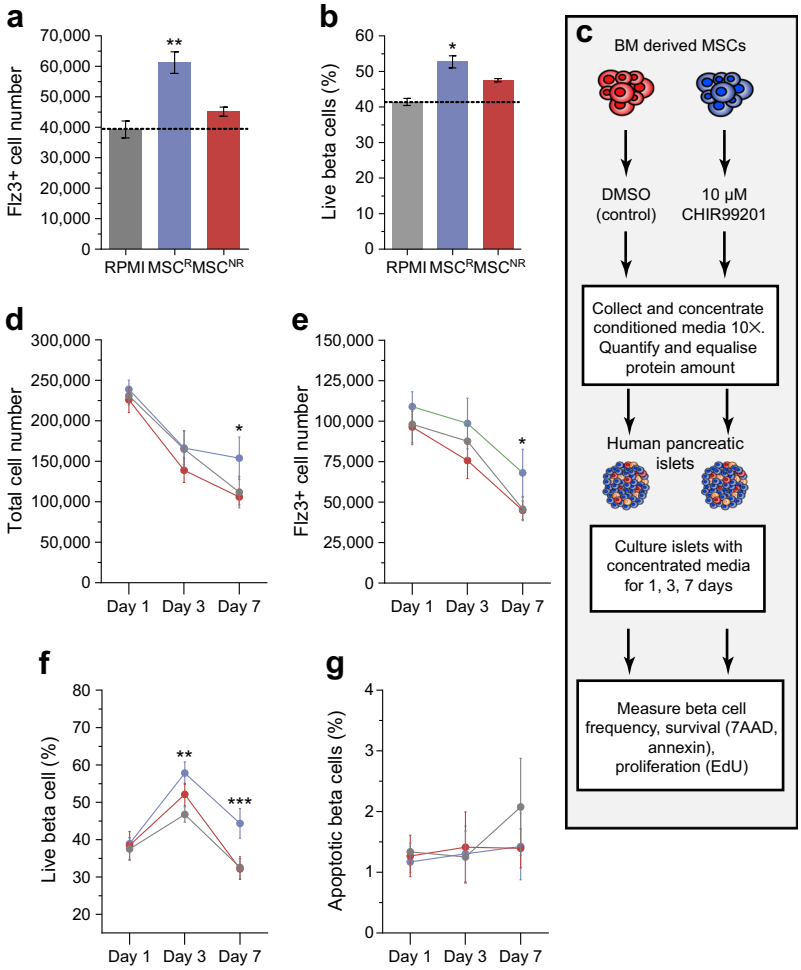

Fig. 5 Culture of human islets in GSK-inhibited CM increased beta cell number. Human islets cultured in CM generated from MSC^{R} showed (**a**) increased beta cell number and (**b**) increased proportion of live beta cells after 7 days of culture. (**c**) Human islets were cultured in CM generated by MSC^{NR} treated with DMSO (basal CM) or CHIR99201 (GSK3i CM). Compared with human islets cultured in basal CM (red), islets cultured in GSK3-inhibited CM (blue) showed increased (**d**) cell number, (**e**) beta cell number and (**f**) proportion of live beta cells at day 7. (**g**) Human islets cultured in MSC^{NR} CM did not alter apoptotic beta cell frequency. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

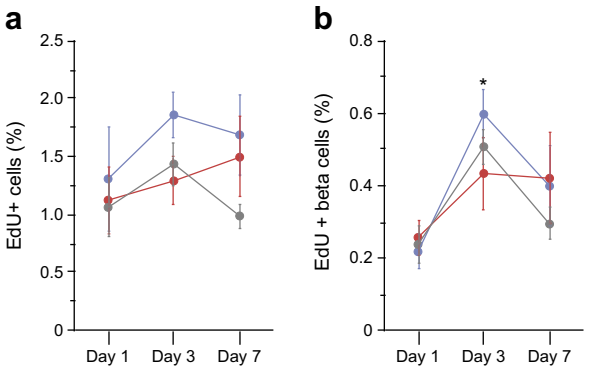

Fig. 6 Culture of human islets in GSK-inhibited CM increased beta cell proliferation. (**a**, **b**) Compared with human islets cultured in basal CM (red), islets cultured in GSK3i CM (blue) showed increased proportion of insulin+/EdU+ beta cells at day 3. (**c**) Islets were obtained through the IIDP from five independent donors with variable weight, BMI and average blood glucose (Avg.BG) values. Data are presented as mean ± SEM. *$p < 0.05$

c

Isolation ID	Age	Sex	Weight (kg)	BMI (kg/m^2)	Avg.BG (mmol/l)
UWHI263R	53	M	107.0	31.10	10.5
H585A	29	F	101.0	35.80	10.8
HP-16206-01	30	M	133.8	35.90	9.8
HP-16250-01	32	F	61.7	24.90	6.4
UWHI267R	37	M	95.0	27.60	9.8

survival and proliferation within cultured human islets in vitro. Thus, we report a central role for Wnt signalling in the establishment of an islet regenerative secretory profile in MSC^R, and improve our understanding of MSC-secreted signals governing islet regeneration. We predict this dataset will aid in the development of future therapies to augment islet regeneration during diabetes.

Functional beta cells secrete proangiogenic proteins responsible for recruiting circulating or tissue resident progenitor cells to islets after damage [34]. However, during autoimmunity or after islet isolation and transplantation these critical functions are likely to be dysregulated, resulting in transient ischaemia that significantly impairs islet function [35]. Increased production of potent proangiogenic factors, such as FGF7, PDGF and VEGFA, by MSC^R underscore the functional capacity to generate a proangiogenic microenvironment. Collectively these proteins potently stimulate human islet vascularisation in vitro [36]. To validate the functional relevance of proangiogenic secretory patterns, MSC^R CM showed increased capacity to induce spontaneous tubule formation by HMVEC compared with MSC^{NR} CM. Interestingly, MSC^{NR} primarily secreted negative regulators of angiogenesis such as FLT1 and FGFR4, which have both been shown to inhibit the signal cascade mediated by VEGFA [19]. We have previously shown that intrapancreatic transplantation of proangiogenic haematopoietic progenitor cells induced islet revascularisation and beta cell proliferation and augmented systemic insulin release in STZ-treated NOD/SCID mice [15]. Therefore, our data suggest MSC^R may also formulate a proangiogenic microenvironment to support beta cell survival and function within endogenous or transplanted islets.

The prevention of proinflammatory states within damaged islets may also be relevant in the context of MSC therapy for type 1 diabetes. The presence of central mediators of inflammation in CM from MSC^{NR}, such as IL-1β, IL-6 and IL-8, suggests that MSC^{NR} may contribute to a proinflammatory cascades. In people with type 1 diabetes, beta cell destruction is initiated by a combination of proinflammatory cytokines, including IL-1β and IL-6 [37], and CXCL family chemokines, leading to recruitment of immune effectors that mediate beta cell destruction [38]. Furthermore, high levels of IL-8 have been linked to elevated NEFA, which can signal inflammatory cascades in the pancreas [39]. In contrast, these cytokines and chemokines were not secreted by MSC^R. Rather, MSC^R secreted cytokines that could potentially reduce inflammation. For example, TGF-β suppresses the secretion of various inflammatory cytokines/chemokines, and induces cytokine secretion patterns that balance local immunity [40]. Stromal cell-derived factor 1 (CXCL12), also upregulated by MSC^R, has been shown to directly promote beta cell survival through the activation of AKT [41]. Collectively, we propose MSC^R generate a niche with reduced inflammation, improving beta cell survival, while MSC^{NR} contribute to a more deleterious proinflammatory microenvironment.

Inadequate beta cell mass leads to hyperglycaemia in both type 1 and type 2 diabetes. Currently, there is significant interest in restoration of beta cell mass through induction of endogenous regenerative mechanisms in situ

using cellular or protein therapies. Our previous transplantation studies suggested that human BM-derived MSCs stimulate an islet regeneration programme with neogenic characteristics. Mice transplanted with MSC^{R}, demonstrated improved glycaemic control via the emergence of small beta cell clusters associated with the ductal epithelium [14]. Several identified effectors may act in synergy to mediate islet regenerative processes. First, MSC^{R} showed increased secretion of EGF previously shown to increase beta cell mass in rodents by stimulating beta cell replication [42]. However, EGF receptor signalling was required for expansion of murine beta cell mass in response to a high-fat diet, but was not crucial for neoislet formation after pancreatic ductal ligation [43]. Second, the CCN family of extracellular matrix-associated, heparin-binding proteins has been shown to modulate cell growth and repair in many tissues by increasing the bioavailability of BMPs, VEGF, Wnt and TGF ligands [44]. More specifically, connective tissue growth factor (CTGF) or CCN2 has been widely studied during beta cell development [45]. Within our dataset, MSC^{R} exclusively secreted CCN5). Previously, we have identified *WISP1* (encoding CCN4) and *WISP2* (encoding CCN5) mRNA as being upregulated in MSC^{R} [15]. Importantly, CCN4 and CCN5 secretion is directly linked to active Wnt signalling. CCN5 also represents a link between insulin and IGF-I regulation in islet function, and CCN5 overexpression leads to increased islet cell growth in vitro [46]. Interestingly, MSC^{NR} exclusively secreted nephroblastoma overexpressed protein or NOV isoform (CCN3), which has been shown to impair beta cell proliferation and inhibit glucose-stimulated insulin secretion in vitro [45]. Thus, we predict modification of the ECM via Wnt-inducible CCN protein regulation is a critical step in islet regenerative processes.

One of the key differences between MSC^{R} and MSC^{NR} was the activation of Wnt signalling. Wnt signalling is highly conserved in primitive MSCs and is involved in multiple developmental processes, including cell proliferation, growth and fate determination. Aly and colleagues have shown that delivery of WNT3A and R-spondin to cultured islets increased the proliferation of adult human beta cells [47]. Our data suggests that MSC^{R} propagate Wnt signalling primarily by autocrine secretion of WNT5A/B ligands with potential regulation by SFRP1. In contrast, MSC^{NR} expressed proteins that inhibit Wnt signalling, namely DKK1 and DKK3. Although more research is required to determine potential effects of Wnt5 signalling on human beta cells, it is clear that MSC^{R} maintain active canonical Wnt signalling and increase secretion of downstream effectors that promote beta cell regeneration. By inhibiting GSK3 activity using CHIR99201, we have shown that the activation of canonical Wnt pathway signals in MSC^{NR} can mimic Wnt signalling allowing accumulation of nuclear β-catenin. Human pancreatic islets grown in media conditioned by MSC^{NR} treated with CHIR99201 showed increased beta cell survival and proliferation during 7 days of culture. Manipulation of Wnt signalling through the inhibition of GSK3 has been previously suggested to directly increase beta cell proliferation. One study treated diabetic neonates with LiCl, a known inhibitor of GSK3, and doubled beta cell mass in rat models of diabetes [48]. Others have inhibited GSK3 using small molecules to regulate islet cell survival and proliferation in vitro [49]. Although, the mechanisms by which inhibition of GSK3 affects beta cell survival and proliferation in vivo is still unclear, our results suggest that activation of Wnt signalling in pancreas-resident stromal cells may have practical application in beta cell regenerative therapies. Nonetheless, activation of Wnt signalling during MSC culture increased the regenerative capacity of MSC^{NR} by altering downstream secretory patterns.

In summary, our data outlines several dynamic and complementary pathways that formulate a regenerative microenvironment applicable to the development of islet expansion therapies for diabetes using MSCs or their secretory products. The proteomic data reported in this study will be used in future studies to characterise functional mechanisms relevant to islet regeneration, or as screening technology to select MSC subpopulations that possess augmented capacity to regenerate islets in situ.

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Data availability Proteomic raw data files that support the findings of this study are available from the corresponding author upon reasonable request.

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Contribution statement MK performed experiments, presented and analysed the data, and wrote and critically revised the manuscript and approved the final version. GIB performed in vivo characterisation experiments, collected and assembled data, and provided critical revision of the manuscript and final approval. SES performed confocal imaging, collected and assembled data, and provided critical revision and final approval of the manuscript. GAL and DAH designed the study, provided financial support and data interpretation, wrote and reviewed the manuscript and provided final approval. DAH is responsible for the integrity of the work as a whole.

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