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RESEARCH

TRAIL induces proliferation in rodent pancreatic beta cells via AKT activation

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Abstract

Strategies to increase functional pancreatic beta cell mass is of great interest in diabetesrelated research. TNF-related apoptosis-inducing ligand (TRAIL) is well known to promote proliferation and survival in various cell types, including vascular smooth muscle and endothelial cells. Correlation between the protective nature of TRAIL on these cells and its proliferative effect is noteworthy. TRAIL's seemingly protective/therapeutic effect in diabetes prompted us to question whether it may act as an inducer of proliferation in pancreatic beta cells. We used rat primary islet cells and MIN6 mouse beta cell line to investigate TRAIL-induced proliferation. Cell viability and/or death was analyzed by MTT, WST-1, and Annexin-V/PI assays, while proliferation rates and pathways were assessed via immunocytochemical and Western blot analyses. Receptor neutralization antibodies identified the mediator receptors. Recombinant soluble TRAIL (sTRAIL) treatment led to 1.6-fold increased proliferation in insulin-positive cells in dispersed rat islets compared to the untreated group, while adenovirus-mediated overexpression of TRAIL increased the number of proliferating beta cells up to more than six-fold. sTRAIL or adenoviral vector-mediated TRAIL overexpression induced proliferation in MIN6 cells also. TRAIL's proliferative effect was mediated via AKT activation, which was suppressed upon specific inhibition. Neutralization of each TRAIL receptor reversed the proliferative effect to some degree, with the highest level of inhibition in death receptor 5 (DR5) blockage in MIN6 cells and in decoy receptor 1 (DcR1) blockage in primary rat beta cells. Thus, TRAIL induces proliferation in rodent pancreatic beta cells through activation of the AKT pathway.

Key Words

- beta cell proliferation
- MIN6 beta cells
- ► TRAII
- rat islet culture

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Introduction

Pancreatic islets are crucial for glucose homeostasis as highly vascularized and innervated mini organs that sense nutrients such as glucose to rapidly secrete vital hormones. This balance is disrupted when islet beta cells are attacked by an autoimmune-mediated destructive

https://jme.bioscientifica.com https://doi.org/10.1530/JME-20-0037 © 2021 Society for Endocrinology Published by Bioscientifica Ltd. Printed in Great Britain mechanism (type 1 diabetes) or display functional defects that may eventually lead to cell death (type 2 diabetes). Replenishing the functional beta cell stores is highly desired for the treatment of both type 1 and type 2 diabetes (Aguayo-Mazzucato & Bonner-Weir 2018, Zhong & Jiang S Kahraman *et al.*

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2019). Advances in the generation of beta-like cells derived from stem cells is promising to establish a functional beta cell mass and is an attractive approach for diabetes treatment (Kahraman et al. 2016). Another strategy to restore beta cell mass is to promote proliferation of preexisting beta cells. Increased metabolic demand leading to increased beta cell mass in conditions such as pregnancy, obesity, or insulin resistance is well acknowledged (Rieck & Kaestner 2010, Remedi & Emfinger 2016, Dirice et al. 2019a). This points out the fact that certain signals within the organism can lead to a boost in beta cell numbers, mainly through proliferation of the existing beta cells, as evidence suggest (Dor et al. 2004, El Ouaamari et al. 2013). Accordingly, several studies identified growth factors, hormones, signaling proteins, and small molecules that have the potential to induce beta cell proliferation (Dirice et al. 2014, 2016, El Ouaamari et al. 2016). Overall, diabetes-related research generally focuses on strategies that aim to compensate for the loss of functional beta cells and to protect/maintain existing beta cells (Aguavo-Mazzucato & Bonner-Weir 2018, Dirice et al. 2019b).

TNF-related apoptosis-inducing ligand (TRAIL) was reported as a new member of the TNF cytokine superfamily in 1995 by two independent groups and has since been thoroughly studied in many disease settings, including diabetes (Wiley et al. 1995, Pitti et al. 1996). With a potential to induce selective apoptosis in a wide variety of transformed cell lines, TRAIL is found on the cell surface as a type II transmembrane protein, which can also be released to form a soluble ligand (Wiley et al. 1995, Shepard & Badley 2009). Of the five receptors that TRAIL can interact in humans, TRAIL-R1/DR4 and TRAIL-R2/DR5 are death receptors that can induce apoptosis, while TRAIL-R3/DcR1, TRAIL-R4/DcR2, and the soluble osteoprotegerin receptor (OPG) are known as decoy receptors, which lack functional domains to trigger apoptosis (LeBlanc & Ashkenazi 2003, Kimberley & Screaton 2004, Holland 2013). Mice, on the other hand, have only one death receptor DR5 that is equally homologous to human TRAIL-R1 and TRAIL-R2, roughly at 60% sequence identity (Wu et al. 1999). Two murine transmembrane decoy receptors homologous to human DcR1 and DcR2 were defined, along with a secreted isoform of rodent DcR2 and another secreted receptor OPG (Harith et al. 2013).

All transmembrane receptors of TRAIL can induce intracellular proliferative/survival pathways. TRAIL induces survival, migration, and proliferation in human and rat primary vascular smooth muscle cells, and survival and proliferation of human vascular endothelial

© 2021 Society for Endocrinology Published by Bioscientifica Ltd. Printed in Great Britain cells (HUVECs), notably protecting both cell types from apoptosis (Secchiero *et al.* 2003, 2004). Besides the vascular system, TRAIL's proliferative effects have also been acknowledged in various other cell types, including human synovial fibroblasts and preadipocytes (Morel *et al.* 2005, Funcke *et al.* 2015).

Expression of TRAIL and its receptors have been detected in many tissues including human pancreas obtained from healthy individuals (Sanlioglu et al. 2008b). The notion that TRAIL has prominent antidiabetic effects came from key studies, such as where quicker and more severe development of diabetes was shown in TRAIL-/rodent models and where TRAIL alleviated metabolic abnormalities in high-fat diet-fed mice (Lamhamedi-Cherradi et al. 2003. Mi et al. 2003. Dirice et al. 2011. Bernardi et al. 2012). Accordingly, we demonstrated significant changes in the expression levels of TRAIL and its receptors in pancreatic islets and acinar cells during the progression of diabetes in non-obese diabetic (NOD) mice, suggesting a functional role for TRAIL and its receptors in pancreas biology (Dirice et al. 2011). We have also shown that TRAIL overexpression in engrafted rat islets prolonged graft survival following transplantation into diabetic rats and significantly delayed graft rejection compared to the control islet-transplanted rats (Sanlioglu et al. 2008a, Dirice et al. 2009).

Thus, based on the strong signs of TRAIL's protective effects in the course of diabetes and the fact that it can induce proliferation in different cell types, we questioned whether TRAIL may have a proliferative effect on pancreatic beta cells, mediating/contributing to its claimed antidiabetogenic properties. According to our results, TRAIL promoted a selective and significant increase in the total number of rat primary beta cells and MIN6 mouse beta cells, through activation of the AKT pathway.

Methods

Primary islet cell culture

Pancreatic islets were isolated from 10–12-week-old female Wistar rats (approximately 225–250 g) provided from Experimental Animals Unit of Akdeniz University, Antalya, Turkey. Rats were kept in a conventional rodent room on a 12 h light: 12 h darkness cycle with access to water and standard rodent chow *ad libitum*. The procedures were reviewed and approved by Akdeniz University Local Committee on Animal Research Ethics and carried out in Experimental Animals Unit and in laboratories of Akdeniz University Center for Gene and

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Cell Therapy. Islet isolation was performed on 5-10 rats at a time and pooled islets were used for each experiment. Isolation procedure was pursued as previously described (Kahraman et al. 2011). Briefly, pancreas was distended in situ with 10 mL cold Liberase RI enzyme solution (Roche, 0.25 mg/mL; prepared in DMEM media without serum) under anesthesia provided via intraperitoneally applied xylazine (40-80 mg/kg) and ketamine (5-10 mg/kg). Pancreas was harvested from surrounding tissues and purified in cold Histopaque 1077 (Roche) after digestion was completed. Isolated rat islets were rested overnight in RPMI medium supplied with 11.1 mM glucose and 10% FBS, and then gently dissociated into single cells by Accutase treatment (Millipore) for 15 min at 37°C via mixing the cells by pipetting every 5 min. At the end of incubation. Accutase was neutralized by addition of 9 mL RPMI medium containing 10% FBS. Cell suspension, counted on a hemocytometer and pelleted by spinning down at 200 g for 5 min, was filtered through a 40 µm filter to remove any undigested aggregates. Dissociated single islet cells were resuspended in RPMI starvation medium containing 5 mM glucose and 2% FBS, seeded in uncoated flat-bottom tissue culture 96-well plates (85,000 cells/ well), and were allowed 24 h to adhere. sTRAIL treatment was initiated at the end of a 24-h starvation period.

MIN6 and A549 cell cultures

MIN6 cells were kindly provided by Dr Jun-Ichi Miyazaki (Osaka University, Japan) to our study group (Miyazaki et al. 1990). They were cultured in high glucose DMEM (Sigma, D5648) supplemented with 3.4 g/L sodium bicarbonate, 1% penicillin-streptomycin, 10% FBS, and 5 µl/L beta-mercaptoethanol at 37°C, 5% CO₂, and 95% humidity. All experiments were done using MIN6 cells with passage numbers between 22 and 30. A549 human lung epithelial cells obtained from ATCC (CCL-185) were cultured in RPMI-1640 media (Sigma, R7755), supplemented with 1% L-Glutamine (Biochrom AG), 2 g/L sodium bicarbonate, 10% FBS (Biochrom AG), and 1% penicillin-streptomycin (Biochrom AG). The cell lines tested negative for mycoplasma.

Treatment with recombinant sTRAIL

MIN6 cells were seeded in 96-well plates (2 \times 10⁵ cells/well/100 µL) and starved for 24 h in high glucose DMEM and 0.5% (wt/vol) BSA before sTRAIL treatment. Recombinant human sTRAIL protein (R&D, 375-TL) was added to the starvation medium (0, 0.1, 1, 10, 100 ng/mL)

and medium with sTRAIL was refreshed after 24 h. Ten percent FBS was used as a positive control group. Dispersed islet cells were seeded in 96-well plates (85,000 islet cells/ well/100 µL) and serum-starved for 24 h in RPMI medium with 5 mM glucose and 2% FBS before treatment with sTRAIL. The starvation medium was refreshed and 0/1/10 ng/mL recombinant human sTRAIL protein (R&D, 375-TL) or 15% FBS (positive control) was added. Media with recombinant protein was refreshed every 24 h.

Assessment of cell viability and cell death

Cell viability and cell death were assessed via several parameters, including MTT assay (AppliChem, A2231), WST-1 assay (Roche, 11 644 807 001), and Annexin-V/ PI staining (Trevigen, 4830-01-K). For the assessment of Annexin-V/PI staining, cells were stained according to manufacturer's instructions. At least five images were taken by Olympus IX81 Fluorescent Microscope and 250 cells were counted per group (n = 3 replicates) for quantification of apoptotic cells (AnnexinV+). WST-1 and MTT assays were performed according to manufacturer's instructions and cell viability was determined via measurement of the optical density at 440 nm (reference 600 nm) and 540 nm (reference 690 nm), respectively (n = 5-8 wells/group).

Adenovirus production and transduction

Adenoviral vectors carrying human TRAIL gene (Ad5hTRAIL) have been amplified as described in detail elsewhere (Griffith et al. 2000, Aydin et al. 2010). For transduction, MIN6 cells were incubated in DMEM with 2% FBS and dispersed islet cells were incubated in RPMI medium with 5 mM glucose and 2% FBS, 24 h prior to the procedure, for synchronization. After PBS wash, Ad5hTRAIL (0, 0.25, 0.5, 1, 2, and 5 µL/mL) was added to cells in DMEM or RPMI with 2% FBS and cells were cultured for 48 h. Multiplicity of infection (MOI) was approximately 500 for 1 µL of Ad5hTRAIL virus applied.

Immunocytochemistry

Cells grown in 96-well plates were fixed in cold methanol (-20°C, 20 min), followed by three PBS washes. Antigen blocking was performed with 5% (v/v) normal donkey serum (Jackson ImmunoResearch, RRID:AB 2337254) in PBS for 1 h at room temperature, and primary antibody was added into each well for overnight incubation at 4°C. The primary antibodies were prepared in antibody diluent (ab64211, Abcam, RRID: not available),

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at 1:100 dilution: guinea pig anti-insulin (Abcam, ab7842, RRID:AB 306130), rabbit anti-glucagon (Abcam, ab18461, RRID:AB_444488), mouse anti-vimentin (Santa Cruz, sc-6260, RRID:AB_628437), mouse anti-Ki67 (BD, 556003, RRID:AB_396287), and goat anti-TRAIL (R&D, AF375, RRID:AB_355334). After three PBS washes, secondary antibody treatment was performed (1:200, in PBS) for 1 h at room temperature, followed by PBS washes. The following secondary antibodies were used: CY2conjugated anti-guinea pig (Jackson ImmunoResearch, 706-226-148, RRID: not available), Texas Red-conjugated anti-rabbit (Santa Cruz, sc-2784, RRID:AB_641184), FITCconjugated anti-rabbit (Jackson ImmunoResearch, 711-095-152, RRID:AB_2315776), Alexa Fluor 594-conjugated anti-mouse (Jackson ImmunoResearch, 715-585-150). Texas Red-conjugated anti-goat (Abcam, ab7123, RRID:AB_955597). Cell nuclei were stained by DAPI and cells were covered by mounting media. Ten images were taken by Olympus IX81 Fluorescent Microscope and approximately 750 cells were counted per group (n=3 replicates) to determine the cellular composition of dissociated rat islets. For the assessment of Ki67+ proliferating primary islet cells, more than 30,000 insulin+ and 2000 vimentin+ cells were counted for each group (n = 3-4 replicate wells) and the experiments were repeated three times independently. For the assessment of Ki67+ proliferating MIN6 cells, 50,000 cells were counted for each group (n = 3-4 replicate wells).

Western blot

Western blotting was performed as reported previously (Kahraman et al. 2015). Briefly, protein extracts (50 µg protein/sample) were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked for 1 h with 2% (wt/vol) BSA and were incubated with antibodies against TRAIL (1:1000, AF375, R&D Systems), phospo-ERK1/2 (Thr202/Tyr204) (1:1000, sc-16982; Santa Cruz Biotechnology, RRID:AB_2139990), ERK1 (1:1000, sc-94; Santa Cruz Biotechnology, RRID:AB_2140110), phospho-p38 MAPK (Thr180/Tyr182) (1:1000, 9211, Cell Signaling, RRID:AB_331641), p38 MAPK (1:1000, 9212, Cell Signaling, RRID:AB_330713), phospho-Akt1/2/3 (Ser473) (1:1000, sc-33437; Santa Cruz Biotechnology, RRID:AB_2225021), Akt1 (1:1000, sc-5298; Santa Cruz Biotechnology, RRID:AB_626658), DR5 (1:1000, AF721, R&D Systems, RRID:AB_2205069), DcR1 (1:1000, AF630, R&D Systems, RRID:AB_355488), DcR2 (1:1000, AF633, R&D Systems, RRID:AB_355491), or beta-actin (1:1000, sc-81178; Santa Cruz Biotechnology,

https://jme.bioscientifica.com https://doi.org/10.1530/JME-20-0037 © 2021 Society for Endocrinology Published by Bioscientifica Ltd. Printed in Great Britain RRID:AB_2223230) at 4°C for 16 h. Proteins were detected with HRP-conjugated anti-goat (1:2000, #1721034, Bio-Rad, RRID:AB_11125144), anti-mouse (1:2000, #1706516, Bio-Rad, RRID:AB_11125547) or anti-rabbit antibodies (1:2000, #1706515, Bio-Rad, RRID:AB_11125142) and visualized with ECL (Roche), and quantified using ImageQuant version 5.1 software.

Neutralization studies

MIN6 cells were seeded in 96-well plates (2 \times 10⁵ cells/ well/100 µL) and starved for 24 h in high glucose DMEM and 0.5% (wt/vol) BSA before treatment. Dispersed islet cells were seeded in 96-well plates (85,000 islet cells/well/100 uL), and serum-starved for 24 h in RPMI medium with 5 mM glucose and 2% FBS before treatment. Neutralizing antibodies to DR5 (AF721, R&D Systems), DcR1 (AF630, R&D Systems), and DcR2 (AF633, R&D Systems) were added to the starvation medium containing 10 ng/mL recombinant human sTRAIL protein. Cells were treated with antibodies in the presence of sTRAIL for 24 h and MTT assay (MIN6) or immunostaining (dispersed islet cells) was performed at the end of treatment to measure cell viability and proliferation. Cells treated only with 10 ng/mL sTRAIL (sTRAIL-only group) were used as control (n = 6 replicate well of MIN6 cells, n = 3 replicate well of rat islet cells). Experiments were repeated three times independently.

Treatment with inhibitors

MIN6 cells were seeded in 96-well plates (2 \times 10⁵ cells/ well/100 µL) and serum-starved for 24 h in high glucose DMEM and 0.5% (wt/vol) BSA before treatment. LY294002 (LC Laboratories #L-7962), PD98059 (LC Laboratories #P-4313), SB203580 (LC Laboratories #S-3400), or DMSO were added to the starvation medium containing 10 ng/mL recombinant human sTRAIL protein. The inhibitors were first dissolved in DMSO at a final concentration of 10 mM, then added at a quantity of 1 µL to 1 mL medium, for a final inhibitor concentration of 10 µM. Our control group 'TRAIL+DMSO' received 10 ng/mL sTRAIL along with 1 µL DMSO in 1 mL medium, which gave us a final concentration of 0.1% DMSO, which is considered noninfluential. This is the same concentration of DMSO also contained in the application medium for the experimental groups. Thus, cells were treated with 10 μ M of each inhibitor in the presence of sTRAIL for 24 h and MTT assay was performed at the end of treatment to measure cell viability. Cells treated with DMSO in the

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presence of sTRAIL were used as the control group (n = 7)replicate well).

Statistical analysis

Statistical analysis was performed by the two-sided unpaired t test or ANOVA as appropriate. All values are means \pm s.E.M. and statistical significance was set at P <0.05. Statistics were performed using Prism7 (Graphpad Software).

Results

Dispersed rat pancreatic islets formed islet-like clusters by 72 h

Pancreatic islets to be used in proliferation studies were isolated from female Wistar rats. Since rodent beta cells



https://jme.bioscientifica.com https://doi.org/10.1530/JME-20-0037 © 2021 Society for Endocrinology Published by Bioscientifica Ltd. Printed in Great Britain are clustered in the center of the islets, we dispersed the islets into single cells to ensure each cell was fully exposed to the recombinant sTRAIL (Steiner et al. 2010). This way, we aimed to ensure higher and more even exposure of all islet cells to TRAIL-mediated effects, without the possible interference of hypoxia formed in the center of many intact islets, and the disadvantage of varying islet sizes. Working on dispersed islet cells also allowed us to test the efficacy of even low concentrations of sTRAIL on all dispersed cells. This also eased transduction of islet cells with adenoviral vectors.

Single islet cells were incubated for 24 h to adhere after dispersion and treated or not treated with sTRAIL or Ad5hTRAIL for 48 h (Fig. 1A). Although we dissociated pancreatic islets into single cells, both treated and untreated islet cells started to accumulate by 48 h and formed islet-like clusters by 72 h after dispersion (Fig. 1B). Islet-like aggregates, formed via spontaneous

Figure 1

Experimental outline and primary rat islet cultures. (A) Schematic representation of the experimental design. Isolated rat pancreatic islets were incubated overnight in RPMI medium containing 11.1 mM glucose and 10% FBS, and then dispersed into single cells and plated in 96-well plates (85,000 cells/well). Cells were allowed to attach for 24 h in starvation medium (RPMI medium containing 5 mM glucose and 2% FBS) and then treated with or without sTRAIL for 48 h in starvation medium. For adenovirusmediated TRAIL overexpression experiments, dispersed cells were transduced with Ad5hTRAIL and assays were performed 48 h later. (B) Representative images of untreated cells at 24, 48, and 72 h after dissociation. Scale bar: 100 μm. (C) Cellular composition of rat pancreatic islet cultures determined by immunostaining. Ten images were taken and approximately 750 cells were counted per group (n = 3 replicates) to determine cellular composition of dissociated rat islets. Immunofluorescent staining of dispersed rat islet cultures for insulin+ beta cells (green), glucagon+ alpha cells (red), and DAPI+ cell nuclei (blue) in combination (upper right). Lower left image shows insulin+ beta cells (green), and vimentin+ fibroblast-like cells (red), and DAPI+ nuclei (blue). Lower right image shows glucagon+ alpha cells (green), vimentin+ fibroblast-like cells (red) and DAPI+ nuclei (blue). Scale bar: 50 µm.

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reaggregation of dispersed islet cells in culture, are claimed to be compatible, even may be superior to intact islets in terms of survival and maintenance of function under hypoxic conditions, also displaying decreased expression of pro-inflammatory genes (O'Sullivan et al. 2010, Annes et al. 2012). We determined the cellular composition of rat islet cell cultures and found a ratio of 73.2% beta cells (insulin+), 16.3% alpha cells (glucagon+), and 5.3% other cell types in the reaggregated islets (only DAPI+). Also, 5.2% of the cultured cells consisted of fibroblast-like cells (vimentin+) (Fig. 1C). Thus, while most cells in the culture were beta cells, ~22% of the cultures consisted of nonbeta islet cells, consistent with the proportions reported before (Steiner et al. 2010). We kept the non-beta cells in culture not to introduce any stress to primary islet beta cells by cell sorting-mediated or drug treatment-mediated elimination of non-beta cells (Phelps et al. 2017).

TRAIL promotes proliferation of primary pancreatic beta cells without inducing apoptosis

Next, we aimed to test for a possible proliferative effect of TRAIL on primary pancreatic beta cells. We first checked whether TRAIL was apoptotic for primary rat islet cells, via Annexin V and Propidium Iodide (PI) stainings (Supplementary Fig. 1, see section on supplementary materials given at the end of this article). Quantification of the number of apoptotic cells (Annexin V+) elucidated no significant difference between the sTRAIL-treated and non-treated groups (10 or 1 ng/mL sTRAIL vs 0 ng/mL sTRAIL, P > 0.05). Apoptotic index was indeed generally, although not statistically significantly, lower in the 15% FBS-treated and also the sTRAIL-treated cells compared to the cells that did not receive any type of treatment (15% FBS: 24.5 ± 5.3%; 10 ng/mL: 27.2 ± 2.8%; 1 ng/mL: $28.9 \pm 3.1\%$ vs 0 ng/mL: $32.8 \pm 5.7\%$).

To test for TRAIL's proliferative effect on primary pancreatic beta cells, dispersed rat pancreatic islets were treated with low or high doses of the recombinant protein (1 and 10 ng/mL) for 48 h and viability was measured using water-soluble tetrazolium salts (WST-1) assay. Cells treated with low dose of sTRAIL (1 ng/mL) and 15% FBS tended to have increased cell viability (1 ng/mL sTRAIL: 1.25 ± 0.15 fold; 15% FBS: 1.19 ± 0.11 -fold vs 0 ng/mL 1.00 ± 0.04 fold, P > 0.05) (Fig. 2A). In parallel, cells were stained for proliferation marker Ki67 and the proliferating beta cells were assessed through detection of insulin and Ki67 double positivity (Fig. 2B and C). Low dose sTRAIL treatment lead to a significant increase in beta cell proliferation compared to the untreated cells (1 ng/mL sTRAIL: $1.56 \pm 0.15\%$ vs

0 ng/mL: 0.97 ± 0.09%; P < 0.05). Adenovirus-mediated overexpression of human TRAIL in dispersed rat islet cells induced the highest level of increase in proliferating beta cells (Fig. 2D and E). Maximum proliferation (more than six-fold) was observed at 0.5 and 1 μ L/mL Ad5hTRAIL doses (0.5 µL/mL: 3.76 ± 0.84%; 1 µL/mL $3.98 \pm 0.48\%$ vs 0 µL/mL: 0.61 ± 0.13%; P < 0.05 and P < 0.01, respectively). Furthermore, to investigate whether the proliferation-promoting effect of TRAIL is specific to the pancreatic beta cells, we measured the proliferation rates of TRAIL-treated non-beta cells in the rat primary islet cultures. Although Ki67 staining was present in some beta cells, it was virtually absent in glucagon+ alpha cells in all groups (Supplementary Fig. 2A). So, we checked the proliferation of the third most abundant cells in islet culture which are vimentin+ fibroblast-like cells. Consistent with the previous findings regarding robust proliferation of fibroblast-like cells derived from islets in *vitro*, we observed $13.3 \pm 1.8\%$ proliferating vimentin+ cells in our rat islet cultures (Gershengorn et al. 2004, Chase et al. 2007, Phelps et al. 2017). Thus, we found a similar yet weaker effect of sTRAIL on fibroblast-like cells only (1 ng/mL: $18.0 \pm 1.0\%$ vs 0 ng/mL: $13.3 \pm 1.8\%$; P = 0.06) (Supplementary Fig. 2A and B).

We also examined the expression of TRAIL in untreated healthy rat pancreatic islet cells and found co-localization of TRAIL with insulin, glucagon, and vimentin (Supplementary Fig. 3), which is consistent with the previous studies including ours demonstrating TRAIL expression in healthy mouse and rat pancreatic islets (Mi et al. 2003, Sanlioglu et al. 2009, Dirice et al. 2011).

TRAIL increased viability and induced proliferation of MIN6 beta cells

To confirm the proliferative effect of TRAIL on pancreatic beta cells, we used the MIN6 mouse beta cell line either by treating the cells with a recombinant soluble TRAIL (sTRAIL) protein or by overexpressing the TRAIL gene via adenoviral vector treatment. In the first experimental setting, MIN6 cells were treated with different doses of sTRAIL (0, 0.1, 1, 10, 100 ng/mL) for 24 and 48 h and cell viability was measured by MTT assay, which is known to be a sensitive detection method of cell proliferation as well as cell viability, measuring the growth rate in terms of a linear correlation between cell activity and absorbance. sTRAIL treatment increased MIN6 cell viability in a concentration-dependent manner in 24 h compared to the untreated cells (0 ng/mL). Maximum response to sTRAIL treatment was with 10 ng/mL and 100 ng/mL

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2.0 В Α Viability (Relative to 0 ng/ml) 1.5 Insulin+ Ki67+ 3 cells (%) 1.0 2 0.5 1 0.0 n -15% FBS 10 mg/ml 10 nolmi 150% FBS ongini 1 nolmi onolmi 1 nolmi sTRAIL sTRAIL С sTRAIL 15% FBS 0 ng/ml 1 ng/ml 10 ng/ml nsulin Ki67 DAPI



Figure 2

Cell viability and proliferation assessment in primary rat islet cell cultures. (A) Viability assessment of primary islet cells by WST-1 assay. Dissociated rat islet cells were starved for 24 h and then treated with different doses of sTRAIL in starvation medium containing 2% FBS for 48 h. Cells treated with 15% FBS were used as control. Cell viability was determined by measuring the optical density (O.D.) at 440 nm (reference 600 nm) (n = 5-6 wells/group). O.D. measurements were normalized to the 0 ng/mL group (Mean ± s.E.M.). P > 0.05 vs 0 ng/mL. P-values were calculated by two-sided unpaired multiple t test and corrected for multiple comparisons using the Holm-Sidak method. (B) Percentage of proliferating beta cells (insulin+Ki67+) 48 h after 0, 1, 10 ng/mL sTRAIL treatment is shown in the graph. Cells treated with 15% FBS were used as control. More than 30,000 insulin+ cells were counted for each group (n = 3 replicate wells) and the experiment was repeated three times with similar results. *P < 0.05, **P < 0.01 vs 0 ng/mL. *P*-values were calculated by two-sided unpaired multiple t test and corrected for multiple comparisons using the Holm–Sidak method. (C) Representative images of proliferating beta cells show insulin+ cells (green), Ki67+ cells (red), and DAPI+ nuclei (blue) in combination. Scale bar: 100 μm. Arrows show proliferating beta cells. (D) Percentage of proliferating beta cells (insulin+Ki67+) 48 h after Ad5hTRAIL transduction to overexpress TRAIL in dissociated rat islet cells (*n* = 3 replicate wells). Results are expressed as mean ± s.ε.м.. *P < 0.05, **P < 0.01 vs 0 μL/mL. P-values were calculated by two-sided unpaired multiple t test with Holm-Sidak method. (E) Representative images of proliferating beta cells show insulin+ cells (green), Ki67+ cells (red), and DAPI+ nuclei (blue) in combination. Scale bar: 100 µm. Arrows show proliferating beta cells.

concentrations at 48 h (10 ng/mL: 1.56 ± 0.15 -fold; 100 ng/mL: 1.54 ± 0.10 -fold; vs untreated: 1.00 ± 0.11 -fold; P < 0.001) (Fig. 3A). Consistent with these findings, MIN6 cells treated with sTRAIL for 48 h displayed a generally higher Ki67 staining, with a statistically significant difference at 10 ng/mL application (10 ng/mL: 77.0 \pm 0.9% vs untreated: 72.8 \pm 1.0%; P < 0.05) (Fig. 3B and C). Second, TRAIL was overexpressed in MIN6 cells by adenoviral vector transduction (Supplementary Fig. 4A and B). A549 lung cancer cell line, which may be

sensitive to TRAIL's apoptotic effects when delivered via adenoviral vectors but not as soluble ligand, was used for comparison (Supplementary Fig. 4D) (Seol *et al.* 2003). TRAIL overexpressing MIN6 cells did not show any signs of cell death, yet in fact displayed significant increase in cell viability at 1 and 2.5 μ L/mL concentrations, compared to the 0 μ L/mL non-transduced cells (1 μ L/mL: 1.17 ± 0.01-fold; 2.5 μ L/mL: 1.12 ± 0.01%; vs 0 μ L/mL non-transduced: 1.00 ± 0.04-fold; *P* < 0.001 and *P* < 0.01, respectively), 48 h post transduction

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(Supplementary Fig. 4E). However, adenoviral vectormediated TRAIL overexpression was toxic to A549 cells at increasing concentrations of Ad5hTRAIL transduction (Supplementary Fig. 4F). These results indicate that TRAIL has an acute and cell-autonomous proliferative effect on MIN6 beta cells.

TRAIL can increase viability and induce proliferation in MIN6 cells via all three transmembrane receptors

We explored the role of TRAIL receptors in mediating the increased viability and proliferation in MIN6 cells induced by TRAIL. Western blot analysis of sTRAIL-treated MIN6 cells displayed varying expressions of DR5, DcR1, and DcR2 receptors on MIN6 cells (Fig. 4A and B). Next, TRAIL receptors were blocked via neutralizing antibodies for investigation of TRAIL receptor involvement in increased viability and proliferation effects exerted by TRAIL. The outcomes were assessed via MTT assay. For this, MIN6 cells were treated with neutralizing antibodies to DR5, DcR1, and DcR2 in the presence of sTRAIL (10 ng/mL) for 24 h. While percentage of viability/proliferation increased with sTRAIL treatment in the sTRAIL-only group compared to the no-sTRAIL group as expected (sTRAIL only: 1.54 ± 0.06 -fold vs no sTRAIL: 1.00 ± 0.06 -fold; P < 0.06-fold 0.001), blocking receptors with anti-DR5, anti-DcR1, and anti-DcR2 antibodies each reversed this effect to some

sTRAIL treatment increased cell viability and proliferation in MIN6 beta cells. (A) Viability assessment of MIN6 cells was done by MTT assay after treatment with various doses of sTRAIL for 24 and 48 h. MIN6 cells were seeded in 96-well plates (2 × 10⁵ cells/well/100 µL) and starved for 24 h in high glucose DMEM and 0.5% BSA before sTRAIL treatment. Cells treated with 10% FBS were utilized as positive control for the assessment of cell viability. O.D. measurements were normalized to the 0 ng/mL group (n = 6 wells/group). *P < 0.05, ***P < 0.001 vs 0 ng/mL. P-values were calculated by two-way ANOVA and corrected for multiple comparisons using Dunnett test. (B) Percentage of Ki67+ proliferating MIN6 cells treated with sTRAIL for 48 h (n = 3-4 wells/group). Approximately 50,000 cells were counted for each group. Results are expressed as mean ± s.E.M.. *P < 0.05 vs 0 ng/mL. P-values were calculated by two-sided unpaired multiple t test and corrected for multiple comparisons using the Holm-Sidak method. (C) Representative images of proliferating MIN6 cells treated with various doses of sTRAIL for 48 h show Ki67+ cells (red), and DAPI+ nuclei (blue) in combination. Scale bar: 50 um.

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degree (sTRAIL+anti-DR5: 1.04 ± 0.06 -fold; sTRAIL+anti-DcR1: 1.12 ± 0.08 -fold; sTRAIL+anti-DcR2: 1.23 ± 0.08 fold; vs sTRAIL only: 1.54 ± 0.06 -fold; P < 0.01, P < 0.01, and P < 0.05, respectively) (Fig. 4C). The highest degree of reversion came along with blockage of DR5, suggesting DR5 as the most influential receptor in MIN6 cells in this regard. On the other hand, the same experiment performed on primary rat islet cells revealed DcR1 as the dominant receptor in sTRAIL-mediated proliferation (Fig. 4D).

TRAIL application activated ERK1/2, p38 MAPK, and AKT kinases in MIN6 cells

TRAIL is known to have the potential to activate kinases such as ERK1/2, p38 MAPK, and AKT, leading to increased of survival/proliferation-related expression genes (Secchiero et al. 2003, Funcke et al. 2015). We tested whether ERK1/2, p38 MAPK, or AKT signaling pathways were activated along with the TRAIL-mediated proliferation of MIN6 cells. Western blot analysis demonstrated that sTRAIL treatment (10 ng/mL) was followed by transiently increased ERK1/2, p38 MAPK, and AKT phosphorylation in MIN6 cells (Fig. 5A). Phospho-ERK1/2 and phospho-p38 MAPK levels peaked at 5–15 min following treatment and then declined toward baseline levels by 60 and 30 min, respectively. Robust increase in AKT phosphorylation was observed 15 min after TRAIL treatment, followed by a





Figure 4

Representative Western blot images showing expressions of TRAIL receptors in MIN6 cells. (A) MIN6 cells were starved for 24 h in high glucose DMEM and 0.5% BSA and treated with 0, 0.1, 1, 10, and 100 ng/mL sTRAIL for 24 h. Cells treated with 10% FBS were also included as a control group. TRAIL receptors DR5, DcR1, DcR2, and loading control beta-actin are shown. (B) Protein levels of DR5, DcR1, and DcR2 were quantified by densitometric analysis and normalized to beta-actin. The data were normalized to the 0 ng/mL controls from the same experiment. Two individual experiments were combined. Results are expressed as mean ± s.E.M. (C) MIN6 cells were seeded in 96-well plates and starved for 24 h before treatment. Neutralizing antibodies to DR5, DcR1, and DcR2 were added to starvation medium containing 10 ng/mL sTRAIL protein. Cells were treated with antibodies in the presence of sTRAIL for 24 h and MTT assay was performed at the end of treatment to measure cell viability. O.D. measurements were normalized to the untreated group (no sTRAIL) (n = 6 replicate wells). (D) Dispersed rat islet cells were serum-starved o/n before treating cells with 10 ng/mL sTRAIL protein and neutralizing antibodies for 24 h. Percentage of proliferating beta cells (insulin+Ki67+) were determined by immunostaining analysis (n = 3 replicate wells). Results are expressed as mean \pm s.e.m.. *P < 0.05, ****P* < 0.001 vs no sTRAIL, †*P* < 0.05, ‡*P* < 0.01, #P < 0.001 vs sTRAIL only. P-values were calculated by two-sided unpaired multiple t test and corrected for multiple comparisons using the Holm-Sidak method. The experiments were repeated three times independently.

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decrease in phospho-AKT levels. These data revealed that ERK1/2, p38 MAPK, and AKT activation accompanied TRAIL-mediated proliferation in beta cells.

To examine the link between ERK1/2, p38 MAPK, AKT activation, and TRAIL-mediated beta cell proliferation, MIN6 cells were treated with sTRAIL in the presence of kinase inhibitors for 48 h. Changes in viability/ proliferation response of beta cells were assessed at the end of treatment by MTT assay (Fig. 5B). We found significant supression of TRAIL-mediated viability/ proliferation in the cells treated with PI3K/AKT inhibitor LY294002. While the ERK1/2 inhibitor PD98059 did not show any significant changes, p38 MAPK inhibitor SB203580 significantly increased viability/proliferation response of beta cells. The inhibitory effects of LY294002 suggest that activation of AKT pathway mediates the beta cell proliferation in response to sTRAIL treatment.

Overall, we demonstrated TRAIL-mediated proliferation in rodent pancreatic beta cells, potentially through each transmembrane TRAIL receptor.

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Discussion

Although predominantly a protective role is attributed to TRAIL in diabetes, many issues are still to be clarified. In this report, we present evidence on proliferative effect of TRAIL on mouse and rat pancreatic beta cells. According to our results, recombinant soluble TRAIL (sTRAIL) treatment and adenovirus-mediated overexpression of TRAIL lead to statistically significant levels of increase in the number of cultured rat primary beta cells and MIN6 mouse beta cells (Figs 2, 3 and Supplementary Fig. 4). Importantly, cell proliferation pathways were activated in TRAIL-treated cells, as was evident from increased phospho-ERK, -p38 MAPK, and -AKT levels following sTRAIL treatment, while AKT inhibition suppressed the proliferative effect (Fig. 5).

TRAIL stood out from the other members of the TNF superfamily shortly after its discovery due to its selective apoptosis induction in many transformed cells, wide expression of its mRNA in human tissues, and lack of toxicity upon systemic administration (Guimaraes *et al.* 2018).

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Overall, having a significant role in tumor defense by natural killer cells and cytotoxic T cells, other effects attributed to TRAIL such as on regulatory T cells, effector T cells, neutrophilic granulocytes, and antigen-presenting cells did not come as a surprise (Beyer *et al.* 2019). The story continued yet on the adverse side as well, when an immunosuppressive, thus, tumor-promoting, effect was claimed within the tumor microenvironment upon TRAIL treatment (Cullen & Martin 2015). TRAIL's diverse effects seem to depend on the cell type, distribution of transmembrane receptors, levels of intracellular molecules that activate or inhibit TRAIL-mediated pathways, and overall on physiological circumstances that shape the type of signals received by the cell (Mert & Sanlioglu 2017, Beyer *et al.* 2019).

Implications for a protective role for TRAIL in diabetes came from a significant study where an earlier and more severe development of type 1 diabetes was reported in mice in the absence of TRAIL (Lamhamedi-Cherradi *et al.* 2003). Accordingly, whole body TRAIL-/- mice presented many diabetes-related complications (Di Bartolo *et al.* 2011). We have previously overexpressed TRAIL in rat pancreatic islets via adenoviral vectors and observed alleviation of insulitis, prolonged normoglycemia, and Western blot analysis of ERK, p38 MAPK, and AKT activation in response to sTRAIL treatment in MIN6 cells. (A) Representative blots show ERK, p38 MAPK, and AKT phosphorylation after sTRAIL treatment. Beta-actin was used as loading control. MIN6 cells were serum-starved for 24 h in high glucose DMEM and 0.5% BSA and stimulated with 10 ng/mL TRAIL for the indicated times. Relative phosphorylation levels of ERK1/2 (Thr202/Tyr204), p38 MAPK (Thr180/ Tyr182), and AKT (Ser473) were quantified by densitometric analysis. Arrows show pAKT and total AKT protein. Western blot quantification of the results were normalized to the 0 min controls from the same experiment. Results from two individual experiments were combined. (B) MIN6 cells were stimulated with 10 ng/mL sTRAIL for 48 h in the presence of 10 µM LY294002, PD98059, or SB203580 dissolved in DMSO (0.1% final concentration in medium). The 'sTRAIL + DMSO' control group contained 10 ng/mL sTRAIL along with DMSO at the same final concentration in medium as the experimental groups (0.1%). MTT assay was performed at the end of treatments to measure cell viability relative to the control group (n = 7wells/group). *P < 0.05 vs DMSO. P-values were calculated by two-sided unpaired multiple t test and corrected for multiple comparisons using the Holm-Sidak method. Results are expressed as mean ± s.E.M.

increased allograft survival upon transplantation of these islets to recipient diabetic rats (Dirice *et al.* 2009). In one other study, we observed a strong suppression of the TRAIL ligand in tissues of NOD mice following treatment with cyclophosphamide, a diabetic agent with a known suppressive effect on T regulatory cells (Dirice *et al.* 2011). In accordance with these findings, TRAIL does not induce apoptosis in primary beta cells, with inhibitors of the TRAIL death pathway presumed to be present in human primary beta cells (Ou *et al.* 2002, Sia & Hanninen 2006).

Among the non-apoptotic functions of TRAIL is the stimulation of survival-, migration-, and proliferation-related pathways in various cell types. TRAIL's proliferative properties is often accompanied by a protective effect against various microenvironmental threats in cells such as vascular smooth muscle cells, HUVECs, human synovial fibroblasts, and preadipocytes (Funcke *et al.* 2015, Secchiero *et al.* 2004). Pancreatic beta cells may also be one of the cell types under protection of TRAIL, apparently setting a barrier against diabetes development, that can easily be eliminated efficiently with blockage of TRAIL in animal models. In cyclophosphamide-applied NOD and NOD/SCID (severe combined immunodeficiency) mice, blocking TRAIL via a recombinant soluble TRAIL

Figure 5

receptor sDR5 following adoptive transfer of diabetogenic T cells lead to a substantially accelerated rate of diabetes development, which was attributed to TRAIL's antidiabetogenic effect, rather than to an increase in beta cell apoptosis (Mi *et al.* 2003). This effect has not been clearly elucidated in the cellular level.

We observed increased viability and significant rate of replication in rat primary beta cells and MIN6 cells treated with sTRAIL or TRAIL-encoding adenoviral vectors (Figs 2, 3 and Supplementary Fig. 4). Although the proliferative effect displayed by the increased FBS concentrations is close to or higher compared to that of sTRAIL on MIN6 cells and dispersed islets, and to that of adenovirusmediated TRAIL overexpression in MIN6 cells, it should be taken into consideration that we tested TRAIL's proliferative action as a single cytokine, vs 10 and 15% FBS contents, containing various essential nutrients and growth factors that facilitate cell survival and proliferation. On the other hand, adenovirus-mediated overexpression of TRAIL in primary beta cells induced up to more than six-fold increase in the number of proliferating beta cells, significantly higher compared to the proliferative effect exerted by 10% FBS. A very high efficiency of adenovirusinduced transduction (70-90%) is expected in dispersed rat islet cells, which was also evident in our study from the highest levels of proliferation with TRAIL-encoding adenoviral vectors (Barbu et al. 2005). TRAIL induced an approximately two-fold increase in the total number of cultured rat vascular smooth muscle cells and also in HUVEC cells. Also, a recently defined proliferative agent on beta cells, SerpinB1, exhibited a two-fold increase in the percentage of insulin+Ki67+ cells (Secchiero et al. 2003, 2004, El Ouaamari et al. 2016). Given the naturally slow proliferative nature of pancreatic beta cells, we believe that up to more than six-fold increased proliferation observed in our study is quite significant.

Importantly, the proliferative effect of TRAIL declines with higher concentrations of the viral vector/TRAIL. This may be due to two seperate factors possibly acting together: inhibition at the receptor level by increasing amounts of TRAIL applied and the emerging cell toxicity with higher concentrations of the virus. It is known that TRAIL may induce up- or downregulation of its own receptors, as was also evident in our study where increased TRAIL doses were associated with substantially lower levels of DR5 and DcR1 receptors in particular (Fig. 4). These are the two receptors we have shown to mediate the proliferative effect of TRAIL in MIN6 cells and the primary beta cells, respectively. Similarly, on the other hand, while the 0.5 and 1 µL/mL concentrations of the viral vector-encoded TRAIL stimulated a high proliferative effect, 2.5 and 5 μ L/mL concentrations were associated with a decreased number of insulin+Ki67+ cells (1 μ L of the viral vector corresponded to approximately 500 MOIs) (Fig. 2D). Although TRAIL is not toxic to islet beta cells, high amounts of the virus is expected to cause toxicity in cells, thus, to an indirect decrease in the proliferative effect. In fact, increased concentration of the viral vector (5 μ L/mL) caused substantial amounts of toxicity in MIN6 cells, which are normally resistant to the apoptotic effect of adenovirus-mediated TRAIL overexpression compared with the A549 cells (Supplementary Fig. 4).

Blockage of TRAIL transmembrane receptors showed us that the proliferative effect of TRAIL was predominantly mediated via DR5 in MIN6 cells, and via DcR1 in primary rat beta cells, while all receptors seemed to mediate such effect at some degree. Besides this expected difference in different cell types, inhibition of the proliferative effect of TRAIL upon receptor blockage was important as an indication that the effect was indeed mediated by TRAIL. Our efforts to study the link between ERK1/2, p38 MAPK, AKT activation and TRAIL-mediated beta cell proliferation revealed that TRAIL's proliferative effects were exerted via the AKT pathway, in compliance with other reports on beta cell proliferation via various different agents (Fig. 5) (Secchiero *et al.* 2003).

MIN6 is defined as a mouse pancreatic beta cell line of insulinoma origin, exhibiting features of glucose metabolism and glucose-stimulated insulin release, similar to the mechanism in normal islets (Ishihara et al. 1993). Yet, it is also considered as a mixed but not a pure beta cell line, as evident from excretion of other islet hormones besides insulin, namely glucagon, somatostatin, and ghrelin; mRNA expression of insulin I and II, somatostatin, proglucagon, ghrelin, and pancreatic polypeptide (PP); and double positivity of insulin and glucagon or somatostatin in some cells (Nakashima et al. 2009). At this point, our observation of proliferation in rat primary pancreatic beta cells following TRAIL application, but not in other islet cell types, clearly displays TRAIL's selective effect on beta cells (Figs 1, 2 and Supplementary Fig. 2). The effect of TRAIL treatment on fibroblast-like cells (vimentin+ cells), which apparently do not interfere with survival or attachment of the endocrine cells, and which are rarely maintained in long-term islet cell cultures, should be investigated further (Supplementary Fig. 2) (Phelps et al. 2017). Although fibroblasts are considered a contamination in primary panreatic islet cultures, and proliferation of which may be debated to have possible

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negative effects, presence of these cells as accessory cells in islet transplantation lead to overexpression of vessel development and beta cell regeneration-related genes and also larger insulin-positive areas, lower cell death, and a faster graft revascularization (Perez-Basterrechea *et al.* 2017).

In conclusion, we display a selective proliferation in pancreatic beta cells upon TRAIL treatment. Our study expands the type of effects exerted by TRAIL within the experimental context of diabetes. One significant point to take into consideration in further studies will be the heterogeneity among beta cells, that is well acknowledged today; whether TRAIL is more effective on certain beta cell subpopulations is yet to be investigated (Dorrell et al. 2016, Da Silva Xavier & Rutter 2020). Results from a variety of other beta cells, particularly human beta cells with efficiency and safety testings, will shape the applicability of TRAIL as a proliferative agent in pancreatic beta cells and is likely to open up a path to new strategies involving in vivo induction of proliferation in existing functional beta cells in diabetic settings and in cells to be transplanted either as allogenic beta cells or autologous cells generated via reprogramming.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/ JME-20-0037.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data availability

Individual data points are shown in the figures. Data are available upon request from the corresponding author.

Author contribution statement

SK designed and performed the experiments, analyzed the data and wrote the manuscript. OY contributed to cell culture and islet isolation experiments. HAA contributed to conceptual discussions and guided in medical relevance. ED contributed to conceptual discussions and edited the manuscript. ADS conceived the idea, designed the experiments,

© 2021 Society for Endocrinology Published by Bioscientifica Ltd. Printed in Great Britain analyzed the data, and wrote the manuscript. All authors have read and approved the final manuscript.

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