Suitability of hepatocyte cell lines HepG2, AML12 and THLE-2 for investigation of insulin signalling and hepatokine gene expression

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Immortal hepatocyte cell lines are widely used to elucidate insulin-dependent signalling pathways and regulation of hepatic metabolism, although the often tumorigenic origin might not represent the metabolic state of healthy hepatocytes. We aimed to investigate if murine cell line AML12 and human cell line THLE-2, which are derived from healthy liver cells, are comparable to hepatoma cell line HepG2 for studying acute insulin signalling and expression of gluconeogenic enzymes and hepatokines. Insulin responsiveness of AML12 and THLE-2 cells was impaired when cells were cultured in the recommended growth medium, but comparable with HepG2 cells by using insulin-deficient medium. THLE-2 cells showed low abundance of insulin receptor, while protein levels in HepG2 and AML12 were comparable. AML12 and THLE-2 cells showed only low or non-detectable transcript levels of \(G6PC\) and \(PCK1\). Expression of \(ANGPTL4\) was regulated similarly in HepG2 and AML12 cells upon peroxisome proliferator-activated receptor \(\delta\) activation but only HepG2 cells resemble the \textit{in vivo} regulation of hepatic \(ANGPTL4\) by cAMP. Composition of the culture medium and protein expression levels of key signalling proteins should be considered when AML12 and THLE-2 are used to study insulin signalling. With regard to gluconeogenesis and hepatokine expression, HepG2 cells appear to be closer to the \textit{in vivo} situation despite the tumorigenic origin.

1. Background

The liver plays a key role in energy homoeostasis by regulation of glucose and lipid metabolism and synthesis of hepatokines. The liver maintains a normal blood glucose level during fasting by breakdown of glycogen and by gluconeogenesis with subsequent release of glucose into the systemic circulation. Postprandial glucose is taken up into the liver and hepatic insulin signalling via the AKT pathway leads to various effects such as glycogen storage, inhibition of gluconeogenesis, promotion of lipogenesis and protein synthesis, and activation of pro-survival pathways. Hepatokines are liver-derived proteins that regulate metabolic pathways in other tissues and have attracted much interest in the last few years since they are in focus for the development of drugs targeting diabetes [1,2]. The comprehensive understanding of the signalling pathways that regulate the metabolic functions in the liver is highly important to target the disturbances in pathological states. Hepatic cell culture
models are indispensable in this context. Undoubtedly primary cells are superior over permanent cell lines since they resemble more the *in vivo* situation. However, the availability of primary hepatocytes is limited, especially human primary hepatocytes are rarely accessible. In addition, the phenotype is unstable and primary cells can only be cultured for a short time span [3,4]. Permanent cell lines have several advantages such as immortality and the possibility to easily interfere with the abundance and activity of potential regulators of metabolic pathways. Cell lines originating from hepatic tumours are immortal but also cells from healthy organs can be artificially immortalized with a variety of methods. In general, liver cell lines are often used for studies on xenobiotic metabolism and hepatotoxicity, and the focus is drawn towards enzyme capacities [5]. In diabetes research, the signalling pathways that regulate hepatic glucose and lipid metabolism are of great interest. The human hepatoma cell line HepG2 is frequently used to investigate aspects of insulin signalling and regulation of gluconeogenic enzymes and hepatokines, and compared them with HepG2 cells. We took into account that the growth media of the three cell lines differ markedly in their insulin content, and used also media with comparable insulin concentrations for the experiments.

2. Results

2.1. Phosphorylation of AKT after acute insulin stimulation

Insulin responsiveness was studied as phosphorylation of Thr-308 and Ser-473 of AKT after acute insulin stimulation for 10 min. HepG2 cells showed significantly increased AKT phosphorylation at both sites after stimulation with 1 nM insulin for 10 min that was further increased with 10 and 100 nM insulin (figure 1).

When AML12 cells were stimulated with insulin in the recommended growth medium containing 850 nM insulin [10], only a marginal increase of phosphorylation was achieved reaching significance for Ser-473 after stimulation with 100 nM insulin (figure 2). Withdrawal of insulin from the culture medium resulted in a reduced basal phosphorylation of AKT (figure 2d). Withdrawal of insulin for 48 h during cultivation did not further increase the response in AKT phosphorylation.

Growth medium of THLE-2 cells also contains high insulin concentrations (800–850 nM insulin) as recommended by...
the manufacturer (www.lgcstandards-atcc.org) and, similarly to AML12 cells, only phosphorylation of Ser-473 of AKT was significantly increased using 100 nM insulin (figure 3). Withdrawal of insulin for 24 h increased the acute effect of insulin and phosphorylation was significantly higher for Thr-308 after stimulation with 10 nM insulin and for Ser-473 after stimulation with 10 and 100 nM insulin (figure 3c, d). In line with AML12 cells, cultivation without insulin for 48 h did not result in a further increase of AKT phosphorylation in THLE-2 cells.

Figure 4 compares ratios of phosphorylated AKT to AKT between the different cell lines and cultivation conditions. When AML12 and THLE-2 cells were grown in their respective growth medium supplemented with insulin, basal AKT phosphorylation was higher when compared with basal levels of AKT phosphorylation in HepG2 cells, but the extent of insulin-induced phosphorylation of AKT was clearly reduced (figure 4a, b). Cultivation in insulin-deficient medium for 24 h reduced basal AKT phosphorylation and increased the extent of AKT phosphorylation on Thr-308 and Ser-473 in AML12 and THLE-2 cells after insulin stimulation to a level that is comparable to HepG2 cells (figure 4c, d). To conclude, when cultivated in media containing equally low insulin concentrations, AML12 and THLE-2 cells showed similar AKT phosphorylation in response to insulin compared with HepG2 cells.

2.2. Protein abundance of IRS1, IRS2 and IR

Protein abundance of key components of the insulin signaling pathway in the cell lines was investigated. THLE-2 cells showed the highest level of insulin receptor substrate (IRS)1 compared with HepG2 and AML12, but a significantly lower signal for the insulin receptor (IR) (figure 5a–c). Notably, withdrawal of insulin for 24 and 48 h significantly increased the level of IR protein in AML12 and a similar trend was observed in THLE-2 cells (figure 5c). Abundance of IRS2 is similar in AML12 and THLE-2 cells and significantly lower in HepG2 cells (figure 5d).
2.3. Expression of gluconeogenic genes

The ability to generate glucose from gluconeogenic precursors is a key feature of hepatocytes and it has been shown that HepG2 cells express the gluconeogenic enzymes glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxykinase (PCK1) and are able to produce glucose [20,21]. We tested if AML12 and THLE-2 cells also express G6PC and PCK1 and respond to cAMP as an important driver of gluconeogenesis. Adenylyl cyclase activator forskolin was used to elevate intracellular cAMP concentrations. When HepG2 cells were starved of serum for 3 h and subsequently stimulated with forskolin for 2 h, a significant increase in G6PC and PCK1 mRNA levels was measured (figure 6a,b). Serum starvation for 24 h before forskolin treatment resulted in a further increase in G6PC and PCK1, both in unstimulated and forskolin-stimulated conditions (figure 6a,b). In AML12 cells, forskolin treatment also induced G6PC mRNA, reaching higher levels after serum starvation for 24 h compared with 3 h (figure 6c). In contrast, in THLE-2 cells G6PC mRNA was at detection limit in unstimulated and stimulated conditions. Measured values of PCK1 were at detection limit in both THLE-2 and AML12 cells. These results suggest that, in contrast to HepG2 cells and primary hepatocytes, these cells show an impaired/lost ability to express gluconeogenic enzymes.

2.4. Regulation of ANGPTL4 expression

Regulation of hepatokines angiopoietin-like protein 4 (ANGPTL4) and fibroblast growth factor 21 (FGF21) is driven by fatty acid-induced peroxisome proliferator-activated receptor (PPAR)α/β activation and, as recently shown, also by cAMP-dependent pathways [22–24]. Since FGF21 mRNA levels were at detection limit in AML12 and THLE-2 cells in all tested conditions, only expression of ANGPTL4 was studied in the three cell lines. Stimulation with PPARα agonist GW501516 for 8 and 24 h increased ANGPTL4 mRNA levels in HepG2 and AML12 cells to a similar extent, with no significant effect in THLE-2 cells (figure 7a–c). Stimulation with PPARα agonist Wy-14643 did not alter ANGPTL4 expression. Similar results were
obtained for the PPARα/δ target gene PDK4 (figure 7d–f), albeit PPARδ-dependent induction was more pronounced in HepG2 compared with AML12, while no significant increase was observable in THLE-2 cells. Stimulation with PPARα agonist showed a weak increase in PDK4 only in AML12 cells. Since PPARα and PPARδ mRNA abundance was comparable in the three cell lines (figure 7g–i), it is unlikely that the non- or weak response to Wy-14643 in HepG2, AML12 and THLE-2 cells was due to low levels of PPARα.

Stimulation of HepG2 cells with forskolin resulted in a significant increase in ANGPTL4 mRNA levels and the increase was even higher when cells were cultured for 24 h without serum (figure 8a). An upregulation of basal ANGPTL4 mRNA levels after serum starvation for 24 h was observed in AML12 cells, but stimulation of these cells with forskolin led to a significant reduction of ANGPTL4 (figure 8b). No regulation in either condition was found in THLE-2 cells (figure 8c). Thus, only HepG2 cells resemble the in vivo regulation of ANGPTL4 by cAMP [24].

3. Discussion

Human primary hepatocytes represent the ‘gold standard’ for studying hepatic insulin signalling and metabolic regulation on the cellular level [25]. Due to their limited availability, high costs when purchased, variation in quality due to variations in preparation and unstable phenotype, permanent cell lines such as HepG2 that are derived from a hepatocellular carcinoma are often used. Immortalized hepatic cell lines that originate from healthy liver are also available and might be of advantage compared with HepG2 cells since their metabolism should present a healthy state. Investigation of the insulin response of hepatic cell lines AML12 and THLE-2 that revealed that substitution of the regular growth medium containing high insulin concentrations with an insulin-deficient medium for 24 h is necessary to achieve an insulin-induced AKT phosphorylation comparable to HepG2 cells. Thus, the high insulin concentrations in the regular culture medium of the manufacturer induced an insulin-resistant phenotype which is observable as reduced extent of AKT phosphorylation. Notably, withdrawal of insulin from the culture medium resulted in a time-dependent increase in the IR protein in AML12 cells. Cultivation in insulin-deficient medium for several passages might therefore induce further changes in the phenotype, probably resulting in a further increase in the insulin responsiveness of AML12 and THLE-2 cells, but this hypothesis needs to be tested with additional experiments. Other groups also reported insulin-induced AKT phosphorylation in AML12 cells when insulin was withdrawn from the culture medium [26,27], but for THLE-2 cells this is the first study.
reporting on insulin responsiveness. In primary hepatocytes, albeit cultured with 1 nM insulin for 16–18 h before stimulation, 0.1 nM insulin is sufficient to induce AKT phosphorylation [28], while 1–10 nM insulin is necessary in AML12 and THLE-2 cells. To conclude, in our experimental conditions, AML12 and THLE-2 cells are not superior over HepG2 cells with regard to AKT activation after acute insulin stimulation.

Analysis of IRS1, IRS2 and IR protein as key components of the insulin signalling cascade revealed differences in the protein amount in the three cell lines. The lower expression level of the IR protein in THLE-2 cells had no pronounced effect on insulin-induced AKT phosphorylation in our study but should be considered when insulin-dependent pathways are studied in this cell line. The band shape of IRS2 in THLE-2 cells looked broader than in samples from HepG2 or AML12 cells, where IRS2 bands were more concise. Differential posttranslational modifications of IRS2 such as phosphorylation, ubiquitinylation, acetylation and glycosylation in THLE-2 cells compared with HepG2 and AML12 cells might be an explanation for the different band shapes [26,27].

In diabetes research, the ability of hepatocytes to perform gluconeogenesis is of great interest since this pathway is dysregulated in insulin resistance and diabetes [29,30]. Gluconeogenic enzymes in HepG2 cells responded in our experimental set-up in a comparable manner to already published results [24]. However, AML12 and THLE-2 cells did not respond to forskolin stimulation to the same extent as did HepG2 cells. We found a similar regulation of \(G6PC\) in AML12 cells in response to forskolin but were not able to detect reliable levels of \(PCK1\) in AML12 cells. This is in some contrast to earlier data where \(G6PC\) and \(PCK1\) transcripts were detected in AML12 cells after stimulation with cAMP and dexamethasone [31] and a low but detectable glucose output was described [26]. However, the authors did not use labelled gluconeogenic precursors to validate whether the

Figure 5. Protein abundance of IRS1, IRS2 and IR in HepG2, AML12 and THLE-2 cells. Cells were grown in their respective growth medium (con) or cultured for 24/48 h without additional insulin. (a) 15 μg protein was separated and checked by western blot for the presence of IRS1, IRS2 and IR. GAPDH was used as loading control. (b–d) Band intensities were normalized for GAPDH (n = 3; mean ± s.d.; *p < 0.05, con versus 24/48 h Ø Ins in AML12 cells; §p < 0.05, con HepG2 versus con THLE-2; ¶p < 0.05, con AML12 versus con THLE-2; ¥p < 0.05, con HepG2 versus con AML12).
observed increase in extracellular glucose was derived from gluconeogenesis. To conclude, the overall ability of AML12 cells to perform gluconeogenesis might be limited and we were not able to detect any reliable G6PC or PCK1 levels in THLE-2 cells.

The expression of hepatic ANGPTL4 was described to be regulated by PPAR \( \alpha \), PPAR \( \delta \) and cAMP [24,32–34]. HepG2 and AML12 cells showed increased ANGPTL4 mRNA levels after PPAR\( \delta \) activation, while there was a minor effect in THLE-2 cells. Also PDK4 as another PPAR\( \delta \) target was increased in HepG2 and AML12 cells and no effect was observed in THLE-2 cells. Of note, the fold increase for PDK4 expression was much higher in HepG2 cells compared with AML12 cells. In contrast to PPAR\( \alpha \) activation, we observed no effects of PPAR\( \alpha \) agonist Wy-14643 on ANGPTL4 expression in all investigated cell lines and only a weak effect on PDK4 in AML12 cells. The non-response of HepG2 and THLE-2 is in contrast to Rakhshanderhoo et al. [32], who stimulated primary hepatocytes from mice and humans with Wy-14643 and measured a significant increase in PDK4. There might be two explanations: a higher concentration of Wy-14643 as we used might be necessary [32], and permanent cell lines might not react to Wy-14643 stimulation [35] as primary hepatocytes do. The mRNA levels of PPAR\( \alpha \) are similar between mouse and human [32]. In our investigated cell lines, PPAR\( \alpha \) levels were also similar and comparable to PPAR\( \delta \). Therefore, weak expression of PPAR\( \alpha \) is not the explanation for the marginal effect of Wy-14643 on the cells.

The cAMP-dependent regulation of hepatic ANGPTL4 expression has been suggested based on in vivo data and was described in HepG2 cells [24] and in brown and white adipocytes [36]. We only observed higher ANGPTL4 mRNA levels after forskolin stimulation in HepG2 cells, but not in AML12 or THLE-2 cells. Again, the two hepatocyte cell lines derived from healthy liver are not closer to the in vivo regulation as compared with HepG2 cells.

In summary, our data suggest that the regular culture conditions with high insulin concentrations generated an insulin-resistant phenotype in AML12 and THLE-2 cells. Although insulin withdrawal from the culture medium resulted in increased insulin responsiveness, AML12 and THLE-2 cells are not superior to HepG2 cells when insulin signalling is studied. Depending on the scientific question, it might be necessary to consider the different expression levels of the insulin receptor and its substrates. For studies on gluconeogenic gene and hepatokine expression, AML12

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**Figure 6.** Expression of gluconeogenic genes in HepG2 and AML12 cells. (a,b) HepG2 cells were deprived of serum for 3 h or for 24 h before stimulation with 20 \( \mu \)M forskolin (FSK) for 2 h. RNA isolation and cDNA synthesis followed qPCR to measure mRNA abundance of G6PC (a) and PCK1 (b). (c) AML12 cells were grown in normal growth medium and deprived of serum for 3 h before stimulation with 20 \( \mu \)M FSK for 2 h. A subset of cells was cultured in insulin-/serum-deficient medium as indicated before the stimulation with 20 \( \mu \)M FSK for 2 h. Expression of G6PC was measured with qPCR. (a–c) Gene of interest was normalized to the housekeeper gene TBP and expression levels are shown as fold change over control (\( n = 3; \) mean \( \pm \) s.d.; *\( p < 0.05, \) con versus FSK; #\( p < 0.05, \) con versus con or FSK versus FSK).
and THLE-2 cells appear not suitable since their expression profile did not resemble the in vivo situation.

4. Material and methods

4.1. Materials

GW501516 was obtained from Santa Cruz whereas Wy-14643 was purchased from Sigma Aldrich. All phosphatase inhibitor components were from Sigma, except sodium-ortho-vanadate, which was purchased from Applichem.

4.2. Cell culture

All cell lines were directly obtained from the manufacturer and were cultivated at 37°C with 5% CO₂. HepG2 cells (ACC 180) were purchased from the DSMZ (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures) and AML12 (CRL-2554™) and THLE-2 (CRL-2706™) cells were from ATCC. HepG2 cells were cultured in RPMI1640 (Lonza) supplemented with 10% FCS (Biochrom), 100 U ml⁻¹ penicillin/streptomycin (Lonza) and 4 mM glutamine (Lonza). For propagation of cells 15 cm cell culture dishes (TPP) were used, and experiments were performed in 6-well plates (TPP). AML12 cells were grown in a 1:1 mixture of DMEM high glucose (Thermo Fischer)/Ham’s F-12 with 1-glutamine (Lonza) and supplemented with 10% FCS, 15 mM HEPES (Carl Roth), 40 ng ml⁻¹ dexamethasone (Sigma Aldrich), 0.005 mg ml⁻¹ (850 nM) human recombinant insulin (Sigma Aldrich), 5 ng ml⁻¹ sodium selenite (Sigma Aldrich) and 0.005 mg ml⁻¹ transferrin (Sigma Aldrich). AML12 cells were propagated in T-75 flasks (Greiner) and experiments were carried out in 6-well plates. For the cultivation of THLE-2 cells the BEGM Bullet Kit (CC-3170) from Lonza was used. The Bullet Kit contains BEBM
Basal Medium and supplements. The final growth medium consists of the following: BEBM supplemented with 10% FCS, bovine pituitary gland extract, hydrocortisone, epidermal growth factor (EGF), insulin, triiodothyronine, transferrin, retinoic acid, 6 ng ml$^{-1}$ human recombinant EGF (Sigma Aldrich) and 80 ng ml$^{-1}$ o-phosphorylethanolamine (Sigma Aldrich). Measurement of insulin in the complete normal growth medium for THLE-2 cells revealed a concentration from 800–850 nM of insulin. THLE-2 cells require a special coating medium that consists of the following: RPMI1640 without glutamine supplemented with 0.01 mg ml$^{-1}$ bovine serum albumin, (heat shock fraction, Sigma), 0.03 mg ml$^{-1}$ type I collagen from bovine skin (Sigma) and 0.01 mg ml$^{-1}$ fibronectin from human plasma (Sigma). THLE-2 cells were cultured in T-75 flasks and experiments were performed in 6-well plates; 4.5 ml of coating medium for a T-75 flask and 1 ml of coating medium for one 6-well plate were applied and culture dishes were incubated overnight in the incubator. Before seeding the coating medium was aspirated.

For experiments in insulin-deficient medium, AML12 and THLE-2 growth medium was prepared as described but no insulin was added to the medium. Therefore, minimal amounts of insulin in the insulin-deficient medium are derived from the insulin that is present in serum.

4.3. Stimulation

Serum was withdrawn from the culture medium of all three cell lines for 3 h before acute stimulation with 1, 10 or 100 nM insulin for 10 min. AML12 and THLE-2 cells were grown either in normal growth medium containing the recommended amount of insulin, or were cultured for 24 and 48 h without additional insulin. When forskolin was used to stimulate cells, they were cultured without serum for 3 or 24 h and in the case of AML12 and THLE-2 cells insulin was also withdrawn for 24 h before stimulation with 20 μM forskolin for 2 h. Stimulation with PPAR agonists was performed in cells grown in their respective growth medium with all recommended additives including insulin. PPAR agonists were used with a concentration of 1 μM and incubation times were 8 or 24 h. All independent experimental replicates were performed as technical duplicates.

4.4. Cell lysis, SDS-PAGE, western blot, protein detection

To harvest samples, cells were washed with cold PBS and lysed in RIPA buffer (25 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton-X-100, pH 7.6)
supplemented with phosphatase inhibitors (100 mM sodium fluoride, 50 mM sodium pyrophosphate, 100 mM sodium ortho-vanadate and 100 mM β-glycerophosphate) and Complet EDTr Min EDTA-free Protease Inhibitor (Roche). Samples were centrifuged for 5 min at 20 000g at 4°C and protein concentration was determined using a BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. 15–20 μg protein was separated on 5–15% reducing gradient gels (running buffer: 200 mM glycerol, 0.1% SDS, 25 mM Tris) and transferred to PVDF membranes with semi-dry western blot. After blocking unspecific binding sites, membranes were incubated overnight with primary antibodies: p-Thr-308, rabbit polyclonal, Cat. 9275, Cell Signaling; p-Ser-473, rabbit polyclonal, Cat. 9271, Cell Signaling; AKT, mouse monoclonal, Cat. 8245, Abcam; IRS1, rabbit polyclonal, Cat. 3089, Cell Signaling; IRS2, rabbit polyclonal, Cat. 3025, Cell Signaling; and insulin receptor protein chain β, rabbit monoclonal, Cat. 3025, Cell Signaling. Membranes were incubated overnight with primary antibodies: p-Thr-308, rabbit monoclonal, Cat. 3025, Cell Signaling; AKT, mouse monoclonal, Cat. 610861, BD Biosciences; GAPDH, mouse monoclonal, Cat. 8245, Abcam; IRS2, rabbit polyclonal, Cat. 3089, Cell Signaling; and insulin receptor protein chain β, rabbit monoclonal, Cat. 3025, Cell Signaling. Membranes were washed 3 × 15 min before incubation with secondary antibody for 2 h at room temperature. Secondary antibodies were all from LI-COR: IRDye® 680LT donkey anti-rabbit IgG (H+L), Cat. 926-68023; IRDye® 800CW donkey anti-mouse IgG (H+L), Cat. 926-32212; IRDye® 800CW goat anti-rabbit IgG (H+L), Cat. 926-32211; and IRDye® 680RD goat anti-mouse IgG (H+L), Cat. 926-68070. Membranes were scanned with the Odyssey Western Blot Scanner from LI-COR and analysed with IMAGE STUDIO LITE (LI-COR).

4.5. RNA extraction, cDNA synthesis and quantitative real-time PCR

RNA was isolated by using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. cDNA was transcribed from 0.5–1 μg RNA using Transcripter First Strand cDNA Synthesis Kit (Roche). qPCR was performed in a 96-well format in a LightCycler 480 (Roche) with QuantiFast SYBR Green PCR Mix (Qiagen). By purifying PCR product with MiniElute PCR Purification Kit (Qiagen), standards for each primer were generated. Standard concentrations ranged from 5 ng μl⁻¹ to 0.5 ng μl⁻¹ and qPCR data were analysed using the LIGHTCYCLER®480 software. All QuantiTect Primer Assays were from Qiagen: human ANGPT14 (Cat. QT00003661), human G6PC (Cat. QT00031913), human PCK1 (Cat. QT00001197), human TBP (Cat. QT00000721), human PDK4 (Cat. QT00003325), human PPARA (Cat. QT00017451), human PPARD (Cat. QT00078064), mouse Angpt14 (Cat. QT00139748), mouse G6pc (Cat. QT00114625), mouse Pck1 (Cat. QT00153013), mouse Tbp (Cat. QT00198443), mouse Pdk4 (Cat. QT00157248), mouse Ppar (Cat. QT00137984) and mouse Ppar (Cat. QT00166292). In this paper only the human abbreviation for the respective gene is used.

4.6. Statistical analysis

Two-tailed unpaired student’s t-test was used to compare two groups and p < 0.05 was considered statistically significant.

Data accessibility. All relevant data are included in the paper.

Authors' contributions. S.S. performed the experiments and analysed data. H.-U.H. conceived and interpreted the data. C.W. conceived, analysed and interpreted the data and wrote the manuscript. S.S.E. conceived experiments, analysed and interpreted data and wrote the manuscript.

Competing interests. We have no competing interests.

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