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Artemether and aspterric acid induce pancreatic α cells to transdifferentiate into β cells in zebrafish

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Author contributions

M.L. and Y.Q. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. M.L., Y.Q. and J.J. designed the study. J.J., Q.K. and S.L. performed key experiments. M.L., Y.Q. J.J. and Y. S. participated in the planning of the work and the interpretation of the results. J.J. drafted the manuscript. M.L., Y.Q., J.J., and F.S.W. revised the paper.

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Bullet point summary

What is already known

- Pancreatic α cells can be induced to convert to β-like cells after experimental compound treatments or gene manipulation.
- Whether α cells convert to β-like cells, when stimulated by artemether and GABA treatment, has previously been controversial.

What this study adds

- Artemether, but not GABA treatment can induce α-cell to β-like cell transdifferentiation, shown by insulin induction and glucose-lowering properties.
- Aspterric acid was newly identified to have similar activity to artemether.

Clinical significance

- The results confirmed the feasibility of α cell to β-like cell transdifferentiation as a therapeutic strategy for diabetes.

ABSTRACT

Background and Purpose: Recently, the anti-malarial drug, artemether, and the neurotransmitter γ-aminobutyric acid (GABA) were identified to convert α cells into β-like cells *in vivo*. However, some of these observations were challenged by other studies. To help address the controversy, we took advantage of zebrafish as a model to perform this study.

Experimental Approach: Firstly, we performed a small molecule screening for artemether and its skeleton analogs. Secondly, we used the Cre-LoxP system for lineage tracing to indicate the conversion of α cells into β cells *in vivo*. The stable transgenic ins2:eGFP αTC1-6 cell line were used for evaluation of α cell transdifferentiation *in vitro*. We further used multiple zebrafish transgenic and mutation lines to demonstrate β-cell differentiation, β-cell ablation and α-cell hyperplasia in this study.

Key Results: We showed that artemether and another sesquiterpene, aspterric acid, induced α cell transdifferentiation into β cells, both in zebrafish as well as using αTC1-6 cells. Furthermore, these two compounds also converted α cells into β cells when β cells were lost or α cells were hyperplastic in zebrafish. Unlike the previous report, the conversion of α cells to β cells was mediated by increasing *Pax4* expression, but not suppression of *Arx* expression.

Conclusions and Implications: Our data suggest that in zebrafish and αTC1-6 cells, both artemether and aspterric acid induce α cell transdifferentiation. Our data, along with those of Li et al. (2017), suggested that artemether and aspterric acid were able to induce α cell transdifferentiation, at least in zebrafish and αTC1-6 cells.

Keywords: α cells; β cells; Transdifferentiation; Zebrafish; Artemether; Aspterric acid.

Abbreviations:

Arx	Aristaless related homeobox
dpf	days post fertilization
EdU	5-ethynyl-2-deoxyuridine
ESCs	embryonic stem cells
GABA	γ-aminobutyric acid
Gcga	Glucagon a
Gcgr	Glucagon receptor
GLP-1	Glucagon-like peptide-1
htBid	human truncated Bid
Ins2	Insulin 2
iPSCs	pluripotent stem cells
Mnx1	Motor neuron and pancreas homeobox 1
Mafa	MAF BZIP Transcription Factor A
Neurod1	Neuronal Differentiation 1
Pax4	Paired Box 4
Ucn3	Urocortin 3

1. INTRODUCTION

Diabetes is characterized by hyperglycemia that results from insulin deficiency, insulin resistance, or a combination of both (Weir, Gaglia & Bonner-Weir, 2020). Insulin is secreted by pancreatic β cells of the endocrine islets, and either a decrease in β-cell mass or β-cell dysfunction result in insulin deficiency (Eizirik, Pasquali & Cnop, 2020). In type 1 diabetes (T1D), it is clear that insulin deficiency is due to autoimmune destruction of β cells by a targeted lymphocyte attack (Boldison & Wong, 2016). In type 2 diabetes (T2D), the insulin deficiency can be caused by β-cell death or loss of their identity (Eizirik, Pasquali & Cnop, 2020). Therefore, restoration of β-cell mass is a promising strategy for diabetes therapy. Thus far, approaches for restoration of functional β-cell mass, have included transplantation of pancreas or islets, transplantation of insulin-producing β-like cells derived from human stem cells, and increasing endogenous β cell regeneration. Although whole pancreas and pancreatic islet transplantation have been established for treatment of T1D (Harlan, Kenyon, Korsgren, Roep & Immunology of Diabetes, 2009), the scarcity of donor pancreata and requirement for chronic immunosuppression limit their clinical application. Other strategies, using human embryonic stem cells (ESCs) or pluripotent stem cells (iPSCs), which can be reprogramed into insulin-producing β-like cells are another possibility, but safety and efficacy issues are still of concern (Nair, Tzanakakis & Hebrok, 2020; Shahjalal, Abdal Dayem, Lim, Jeon & Cho, 2018).

Over the last few years, β-cell regeneration, including stimulation of β-cell replication, induction of progenitor differentiation, and conversion of exocrine cells or other endocrine cells to β cells, have been of considerable interest. Exocrine and endocrine cells have plasticity and can be reprogrammed to become insulin-producing cells (Migliorini, Bader & Lickert, 2014). Since the pancreatic α and β cells arise from a common multipotent progenitor, and the mass of α cells expands in diabetes, α cells are a desirable target for insulin-producing cell reprogramming (Thorel et al., 2010; Unger & Cherrington, 2012; Ye, Robertson, Hesselson,

Stainier & Anderson, 2015). Ectopic expression or knockout of several master regulatory transcription factors that are involved in normal pancreas development, such as Pdx1, Pax4, and Arx, can induce α cell transdifferentiation into β cells (Collombat et al., 2009; Courtney et al., 2013; Matsuoka et al., 2017; Yang, Thorel, Boyer, Herrera & Wright, 2011). Moreover, small molecules (BRD7389, Resveratrol and dapagliflozin) or peptides (GLP-1) can stimulate α to β cell conversion (Fomina-Yadlin et al., 2010; Lee, Lee, Choung, Jung & Jun, 2018; Wei et al., 2020; Xie, Sinha, Singh, Li, Han & Yen, 2013; Zhang et al., 2019). In 2017, two groundbreaking studies reported that the anti-malarial drug artemether, followed by the neurotransmitter γ-aminobutyric acid (GABA), induced α-to-β-like cell conversion *in vivo* (Ben-Othman et al., 2017; Li et al., 2017), in zebrafish, rodents and *ex vivo* pancreatic islets. Both compounds activated the γ-aminobutyric acid (GABA) signaling pathway and reduced the expression of *Arx,* a crucial transcription factor that directs α cell fate. However, two later studies in mice challenged some of these findings (Ackermann, Moss & Kaestner, 2018; van der Meulen et al., 2018). Here, irrespective of whether the mice were treated with artemether and GABA *in vivo* or cultured murine islets were treated *in vitro* (van der Meulen et al., 2018), they found no evidence of α-to-β cell transdifferentiation. On the contrary, some of their results revealed that mouse islets incubated with artemether possessed the characteristic of de-differentiation and suppressed glucose-stimulated insulin secretion (van der Meulen et al., 2018).

Artemether, belonging to the terpenoid group of compounds, has been demonstrated to have anti-diabetic effects (Basha & Sankaranarayanan, 2014; Chen, Li, Wu, Ren & Zhang, 2008; Kang et al., 2009; Liu et al., 2020; Perez Gutierrez & Baez, 2020). To explore whether terpenoid anti-diabetic compounds exert their effect through induction of β-cell regeneration, especially α to β cell transdifferentiation, we took advantage of the pharmacological tractability of zebrafish. We screened 15 different sesquiterpenes or diterpenoids that have been implicated

in β-cell regeneration, and also included GABA in this screening. Using a series of pharmacological and genetic analyses, we have demonstrated that artemether and aspterric acid, whose skeletons are similar, were able to induce α to β cell conversion in zebrafish. However, although GABA increased β-cell mass, through induction of precursor cell differentiation, α to β cell transdifferentiation did not occur.

2. MATERIALS AND METHODS

2.1 Zebrafish lines and maintenance

Zebrafish (*Danio rerio*) were raised in an aquaculture system (Haisheng, China) on a 14:10-h light-dark cycle at 28°C. The larvae used for experiments were generated by natural crossing and the fish were raised in the same temperature, in an embryo-rearing solution, and staged according to Kimmel et al (Kimmel, Ballard, Kimmel, Ullmann & Schilling, 1995). The following previously published transgenic lines were used: *Tg(gcga:GFP)* (Zecchin et al., 2007), *Tg(Ins:H2BmCherry)* (Maddison & Chen, 2012), *Tg^{BAC}(Neurod1:EGFP)* (Obholzer et al., 2008), *Tg(mnx1:eGFP)*(Yang, Covington & Chen, 2020), *Tg(Ins:htBid$^{TE\text{-}ON}$; LR)* (Li, Maddison, Page-McCaw & Chen, 2014), *Tg^{BAC}(arx:Cre;LC)* (Helker et al., 2019), *Tg(ins:loxP:mCherry STOP:loxP:H2B-GFP)* (Helker et al., 2019). *gcgra$^{-/-}$;gcgrb$^{-/-}$* double mutant fish (referred as *gcgr$^{-/-}$* henceforth)(Li, Dean, Zhao, Nicholson, Powers & Chen, 2015). All experiments involving animals were performed according to local guidelines and regulations of Xiamen University Institutional Animal Care and Use Committee (Protocol XMULAC20160089, 10 March 2016).

2.2 Treatment with small molecules, and screening in zebrafish

14 sesquiterpenoids or diterpenoids are natural products, extracted and identified by our laboratory, the detailed chemical structures of which are listed in supplemental Figure.S1.The active compound aspterric acid, isolated from *Penicillium polonicum,* and the procedures for isolation were performed as described in our previous study (Liu et al., 2020). Its detailed chemical characterization is shown in supplemental Figures S2-S4. Other compounds used in the study were: artemether (A107447, Aladdin), GABA (A010191, Energy Chemical), doxycycline hyclate (324385, Sigma-Aldrich), and tebufenozide (31652, Sigma-Aldrich). All compounds were prepared as a 1,000X stock solution and stored in Eppendorf tubes at -20°C. Except for GABA (10mM), which was dissolved in sterile water, the others were dissolved in DMSO, at a concentration of 10 mM. Before small molecule compound treatment, 3 dpf (days post fertilization) embryos were collected into 24-well plates with 0.3X Danieau solution. The compounds were added to the solution to give the appropriate final concentration and refreshed every 24 hours. After 72 hr treatment, larvae were harvested and fixed.

2.3 Imaging and fluorescence positive cell counting

Counting of fluorescence positive cells was performed as described previously (Li, Page-McCaw & Chen, 2016). In brief, larvae were fixed in 4% paraformaldehyde at 4℃ overnight, and then were washed with 1X PBS plus 0.1% Tween-20 (PBST) and flat mounted in Aqua-Mount (Richard-Allan Scientific) with their right side facing the coverslip. The α-cell and β numbers were counted according to the GFP or nuclear mCherry signal under a Zeiss AxioImager A1 microscope (Carl Zeiss, Jena, Germany). For double positive cell analysis, the mCherry or eGFP signal were collected using a Leica SP8 confocal microscope (Leica, Wetzlar, Germany) with z-stacks. Then the confocal projections were analyzed with ImageJ software V1.53c (ImageJ, RRID:SCR_003070). Briefly, the GFP channel and RFP channel were merged in z-stacks in the software, and the double-positive cells were counted manually, slice by slice.

All the counting was reviewed by a blinded reviewer.

2.4 Lineage Tracing

Tg^{BAC}(arx:Cre) zebrafish were crossed with *Tg(ins:loxP:mCherry STOP:loxP:H2B-GFP)* zebrafish, and the double positive larvae *Tg^{BAC}(arx:Cre); Tg(ins:loxP:mCherry STOP:loxP:H2B-GFP)* were picked. These larvae were then treated with 10 μM artemether, GABA or aspterric acid for 72 hr from 3 dpf to 6 dpf, and then they were fixed and z-stacks were imaged with Leica SP8 confocal microscope (Leica,Wetzlar, Germany).

2.5 Cell culture and establishment of stably transfected cell lines

The mouse pancreatic α cell line, αTC1-6 (ATCC Cat# CRL-2934, RRID: CVCL_B036), was grown in low-glucose DMEM supplemented with 10%FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin, MEM non-essential amino acids and maintained in a humidified incubator with 95% air and 5% CO2 at 37 °C.

0.5 kb mouse insulin 2 (ins2) promoter was amplified by polymerase chain reaction (PCR) using KOD DNA polymerase (AP301, TransGen Biotech). The PCR primers used were as follows: 5'-GACTAGTGGACCATTAAGTGCCTTGCT-3' and 5'-CGACCGG TGGATCACTTAGGGCTGGTGG-3'. The amplified fragment was then subcloned into pcDNA3.1-Puro-CAG-eGFP at SpeI and AgeI sites, resulting in a pcDNA3.1-Puro-ins2-eGFP vector. αTC1-6 cells were transfected with Liposomal Transfection Reagent (40802, YeaSen) according to the manufacturer's instructions. The transfected cells were selected under 2.5μg/mL puromycin (A610593-0025, Sangon Biotech) for 1 week, and further confirmed by PCR.

2.6 Small molecule compound treatment and measurement of fluorescence intensity in αTC1-6 cell line.

The stable pcDNA3.1-Puro-ins2-eGFP αTC1-6 cells were grown on glass coverslips in a standard environment in 24-well plates. Compounds were added to the medium at the appropriate final concentration. After 72 hr incubation, cells were fixed in 4% paraformaldehyde and imaged with a Leica SP8 confocal microscope using the same criteria. Every sample was analyzed in at least 5 fields of view, and the intensity of eGFP fluorescence was measured using ImageJ software V1.53c (ImageJ, RRID:SCR_003070). In general, the region of interest (ROI) was selected by threshold, and the mean gray values (the ratio of integrated density to area) were calculated automatically.

2.7 EdU staining and whole mount immunofluorescence

To identify proliferating β-cells, drug-treated *Tg(ins:H2BmCherry)* larvae were incubated with 1 mM 5-ethynyl-2-deoxyuridine (EdU) at 4 dpf for 48 hr, and EdU was detected using the Click-iT EdU Alexa Fluor 488 Imaging Kit (C0071S, Beyotime) according to the manufacturer's protocol. Z-stack images were collected by a Leica SP8 confocal microscope and Image J software was used to analyze the double positive cells.

For whole mount immunofluorescence, the larval zebrafish of *gcgr*$^{-/-}$*;Tg(gcga:GFP)* were treated with the small molecule compounds for 72 hr and fixed in 4% paraformaldehyde in PBS overnight at 4°C. The larvae were then stained using polyclonal anti-insulin antibody (Agilent Cat# A0564, RRID:AB_10013624) as primary antibody, and Alexa Fluor 594 Goat anti-guinea pig antibody (Thermo Fisher Scientific Cat# A-11076, RRID:AB_2534120) as secondary antibody, using standard techniques (Li, Page-McCaw & Chen, 2016).

2.8 β-cell ablation and free glucose assays

To ablate the β cells, 2 dpf triple transgenic *Tg(Ins:htBid$^{TE\text{-}ON}$;LR); Tg(Ins:H2BmCherry);Tg(gcga:GFP)* larvae were incubated with doxycycline (100μM) and tebufenozide (50μM) for 48 hr in the dark, then incubated in compound-free 0.3X Danieau solution for 24 hr. Starting from 5 dpf, β-cell ablated triple transgenic larvae were treated with 10μM of the different compounds for 48 hr. At 7 dpf, the larvae were fixed and imaged using confocal microscopy, or the total free glucose was measured.

Total glucose was measured using an Amplex Red Glucose/Glucose Oxidase Assay Kit (A22189, Thermo Fisher). A pool of 10 larvae was homogenized in 100 μL of sample buffer. The homogenate was spun at 12,000 rpm, 4°C for 5 min. Free glucose in 10 μL of supernatant (equivalent of one larva) was measured using a GloMax® Discover Microplate Reader (Promega) according to the manufacturer's instruction. At least five pools of each sample were measured.

2.9 Isolation of islets, RNA extraction and quantitative real-time RT-PCR.

After treatment with 10 μM small molecule compounds at 3 dpf for 72 hr, *Tg(gcga:GFP);Tg(ins:H2BmCherry)*, the transgenic larvae were anesthetized and digested in 250 μl collagenase P solution (0.6mg/ml, dissolved in HBSS, Roche) for 5 min at 37°C. The digestion was then stopped by adding 1 ml stop solution (10% FBS in HBSS). The lysate was spun and the pellet resuspended in cold HBSS plus 10% FCS. The suspension was transferred to a petri dish, and the islets picked under Leica M205 FCA fluorescence stereomicroscopy (Leica,Wetzlar, Germany), the islets were pooled and from each sample collected, at least 60 islets were used for RNA extraction.

Total RNA was extracted from islets, larvae and αTC1-6 cell using Trizol Reagents (Thermo Fisher), according to standard procedure. Relative gene expression was analyzed by qRT-PCR, using 2× SYBR Green PCR Master Mix (Lifeint, Xiamen, China) and primers listed in Table

S1. Results were calculated with $2^{-\Delta\Delta Ct}$, and the expression level was normalized to the housekeeping gene β-actin and expressed as relative fold change from control group. For the control group of qRT-PCR, each control value was normalized to the mean value of the control group (mean values shown as 1). For each gene, qPCR was performed in triplicate of each cDNA, from at least 5 biological samples. Primers for quantitative qRT-PCR are listed in Table S1.

2.10 Dual-luciferase reporter assay

A 409 bp mouse *Pax4* promoter was subcloned upstream of the firefly luciferase gene in a PGL6 vector (D2102, Beyotime), resulting in a PGL6-Pax4-P vector. The empty PGL6 plasmid was used as a negative control. PGL6 or PGL6-Pax4-P plasmids were co-transfected with PRL-SV40-C (D2768, Beyotime), which contained an SV40 promoter upstream of the Renilla luciferase gene, into αTC1-6 cells. Lipofectamine™ 3000 (L3000015, Thermo Fisher) was used as the transfection reagent, according to the manufacturer's instructions, and 48 hr after transfection, 10 μM of the different small molecule compounds was added, and the cells were incubated for 72 hr. Finally, luciferase activity was determined using the dual luciferase reporter assay system (RG088S, Beyotime), and the ratio of firefly luciferase to renilla luciferase was calculated.

2.11 Statistical analysis

The data and statistical analysis complied with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018), with the exception that some group sizes for the zebrafish islet cell counts were unequal due to technical difficulties. The experiments were carried out in a randomized manner, and the data analyzed by a blinded reviewer. All the raw data were analyzed with GraphPad prism8 software (GraphPad Software, La Jolla, CA, USA) (GraphPad, RRID:SCR_000306). Results are

presented as mean values ± SEM. No outlying values were excluded from the datasets used for statistical analysis. All data were first subjected to the Shapiro–Wilk normality test to determine whether parametric or non-parametric analyses. If the data were found to follow a Gaussian distribution, parametric tests were performed with two-tailed unpaired Student's t-test (two groups) or one-way ANOVA (three or more groups). The data analyzed by ANOVA were checked for inhomogeneity of variances by Brown-Forsythe test before the *post hoc* tests. Data subjected to ANOVA were followed by Bonferroni *post hoc* tests only when the F value attained $P< 0.05$ and there was no significant inhomogeneity of variances. If the data did not follow a Gaussian distribution, a non-parametric test (Mann–Whitney test to compare two groups, or Kruskal–Wallis test with Dunn's *post hoc* test to compare three or more groups) was performed. In all cases, differences were considered significant when $P<0.05$.

2.12 Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

3. RESULTS

3.1 Artemether and its skeleton analog aspterric acid, increased β-cell generation due to α-cell transdifferentiation in zebrafish.

As artemether, a sesquiterpenoid, stimulates the conversion of α cells into β cells *in vivo* (Li et al., 2017), we investigated 14 other sesquiterpenoids or diterpenoids, including artemether for screening, using zebrafish as a model. We included GABA in the screening, as GABA has also been reported to have similar effects (Ben-Othman et al., 2017). The double transgenic zebrafish *Tg(gcga:GFP);Tg(Ins:H2BmCherry)*, in which α cells are labeled with GFP, driven by a glucagon promoter, and β-cells are labeled with nuclear mCherry, driven by

an insulin promoter, were employed for the assay. Transdifferentiated cells were identified as bi-hormonal (glucagon- and insulin-co-expressing) cells, and were GFP and mCherry double positive (Fig. 1A). After co-incubation with different compounds for 72 hours from 3 dpf, the larvae were imaged and their islets analyzed. As shown in Fig. 1B and 1C, artemether and aspterric acid, but not GABA or other compounds, significantly increased the bi-hormonally positive cells. We also counted the α and β cells after the treatment, and found that artemether, aspterric acid and GABA all significantly increased β cell number, and decreased α cells simultaneously (Fig. 1D and 1E). We then investigated the expression level of insulin and glucagon genes. As shown in (Fig1G-1J), insulin genes (*insa* and *insb*) were significantly upregulated after treated with artemether and aspterric acid, but not GABA. However, glucagon genes (*gcga* and *gcgb*) were unaffected. Taken together, these data suggested that artemether and aspterric acid likely induce α to β cell conversion. The GABA-induced β-cell increase may occur by other mechanisms.

3.2 In vivo lineage tracing and in vitro assays both confirmed that artemether and aspterric acid induced α cell transdifferentiation into β cells

To further verify the results from the screening data, we then applied the lineage tracing approaches for analysis. We crossed the *Tg^{BAC}(arx:Cre)* zebrafish line, in which Cre recombinase was driven by the *arx* promoter expressed in the *arx* positive cells (α cells), to the *Tg(ins:loxP:mCherrySTOP:loxP:H2B-eGFP)* reporter zebrafish line, in which the reporter system was expressed in the insulin positive cells (β cells). When α cells transdifferentiated into β cells, the reporter allele was turned on, and the Cre recombinase excised the mCherry STOP cassette, leading to H2B-GFP expression in the nucleus of these cells (Fig.2A). After co-incubation of these double transgenic larvae with artemether, aspterric acid and GABA for 72 hr, we counted the eGFP-positive cells in the islet. As shown in Fig. 2B and 2C, artemether and aspterric acid, but not GABA, significantly increased the α to β cell transdifferentiation.

These data suggested that artemether and aspterric acid induced newly formed β cells from α cells in zebrafish.

We further tested these compounds using an *in vitro* assay. To evaluate whether these two compounds could induce α-cell transdifferentiation *in vitro*, we generated a stable transgenic ins2:eGFP αTC1-6 cell line, which eGFP was driven by the mouse insulin 2 promoter and inserted in the genome (Fig. 2D). Based on this stable cell line, if the α cells transdifferentiate to β-like cells, the insulin promoter will initiate the expression of eGFP, which can be detected by fluorescence microscopy (Fig. 2D). After treating the ins2:eGFP αTC1-6 cell line with artemether, aspterric acid and GABA for 72 hr, we imaged the treated cells and measured the fluorescence intensity of eGFP. The results indicated that artemether and aspterric acid treatment significantly increased the expression of eGFP in a dose-dependent manner, while the eGFP level of the GABA-treated cells did not change (Fig. 2E-G). These data suggested that artemether and aspterric acid stimulated the αTC1-6 cells to transdifferentiate to β- like cells.

3.3 Artemether and aspterric acid-induced zebrafish β-cell generation was not due to precursor cell differentiation or existing β-cell replication.

Since artemether, aspterric acid and GABA increased the β cell number (Fig.1C), we then investigated whether this increase in number was due to the differentiation of endocrine progenitor cells or the replication of existing β cells. To firstly determine whether these three compounds stimulated differentiation of endocrine precursor cells, we employed the double transgenic zebrafish *Tg^{BAC}(Neurod1:eGFP); Tg(Ins:H2BmCherry)*, in which newly-differentiated β cells in the islet were labeled with eGFP driven by the *Neurod1* promoter, while all β cells were labeled with mCherry. The eGFP^{+}/mCherry^{+} double positive cells were identified as the newly-formed β cells by a process of differentiation. All three compounds increased the total β cells after treating these double transgenic larvae for 3 days (Fig. 3A and

3B). However, neither artemether nor aspterric acid increased the newly-differentiated β cells as there was no change in the $eGFP^+/mCherry^+$ double positive cells, while a small increase in these cells was induced by GABA (Fig. 3A and 3C). Furthermore, we used the *Tg(Mnx1:eGFP);Tg(Ins:H2BmCherry)* zebrafish, in which the mnx1 promoter was active in a subset of the precursors of the newly differentiated β cells (Maddison & Chen, 2012). Similar to results obtained from Tg^{BAC}*(Neurod1:eGFP); Tg(Ins:H2BmCherry)* zebrafish, we saw an increase in $eGFP^+/mCherry^+$ double positive cells in the GABA-treated group, but not in the artemether nor aspterric acid-treated groups (Supplemental Fig. S5). These results indicated that the β-cell generation, induced by artemether or aspterric acid, was not due to precursor cell differentiation, whereas the β-cell generation induced by GABA may have partially come from pancreatic endocrine precursors.

To determine whether β-cell proliferation contributes to induced β-cell increase, we applied EdU staining to indicate the cell proliferation. Artemether and aspterric acid treatment of *Tg(Ins:H2BmCherry)* reporter fish was as described above, and EdU was co-incubated with the compounds for 48 hrs. After the treatment, we counted the EdU positive β cells. As shown in the Fig. 3D and 3E, none of the compounds increased the double positive cells compared with the control group. Taken together, these data indicated that none of the three compounds increased the replication of existing β cells, and β-cell proliferation was not the source of the induced β-cell increase.

3.4 Artemether and aspterric acid induced α-cell transdifferentiation during extreme β-cell loss in zebrafish

To further determine whether artemether, aspterric acid and GABA could promote α-cell transdifferentiation during β-cell loss, we used a zebrafish β-cell ablation model $Tg(Ins:htBid^{TE\text{-}ON};LR)$, in which β cells can be specifically ablated (Li, Maddison, Page-McCaw & Chen, 2014). In this transgenic line, the proapoptotic protein tBid, is expressed

under the control of the tetracycline- and ecdysone-inducible system, and induced tBid could result in the apoptosis of β cells (Fig. 4A). We crossed *Tg(Ins:htBid^{TE-ON};LR)* with *Tg(Ins:H2BmCherry);Tg(gcga:GFP)* zebrafish, and then induced the expression of tBid in these triple transgenic larvae for 48 hr, followed by 24 hr washout. Following this, the β-cell ablated larvae were incubated with different compounds for 2 days, and then their α and β cells were counted (Fig. 4A). Interestingly, artemether and aspterric acid, but not GABA, induced significant restoration of β-cell number (Fig. 4B and 4C). Moreover, these two compounds slightly decreased the α-cell number compared with the DMSO-treated group, although this was not statistically significant (Fig. 4D). Notably, artemether or aspterric acid-treated larvae significantly increased the number of bi-hormonal (glucagon- and insulin-co-expressing) cells compared to the DMSO-treated control (Fig. 4E). Furthermore, we also evaluated the expression level of insulin and glucagon after the triple transgenic larvae were treated with different compounds. As shown in Fig 4F to 4I, the transcription of one of the genes coding for insulin expression (*insa*) was significantly upregulated after the larvae were treated with artemether and aspterric acid. The transcription of one of the genes coding for glucagon (*gcga*) was downregulated by these three compounds. These data indicated that artemether and aspterric acid induced a portion of α cells to transdifferentiate into β cells, when the β cells were ablated.

To further investigate functionality of these restored β cells, we then measured the total free glucose levels of compound-treated β-cell ablated larvae. After the induction of tBid, *Tg(Ins:htBid^{TE-ON};LR)* larvae produced significantly increased total free glucose levels, compared with un-induced larvae (DMSO *vs* control) (Fig. 4J). Most interestingly, after β-cell ablation, the total free glucose level was significantly reduced in the larvae incubated with artemether or aspterric acid (Fig. 4J). These data suggested that the β cells restored by artemether or aspterric acid α-cell transdifferentiation were functional.

3.5 Artemether and aspterric acid induced hyperplastic α cells to transdifferentiate to β cells in zebrafish

Disruption of glucagon receptor (GCGR) signaling is associated with α-cell hyperplasia in many organisms, from zebrafish and mice to humans (Gelling et al., 2003; Kang et al., 2020; Li, Dean, Zhao, Nicholson, Powers & Chen, 2015; Yu, 2018). Induction of these supernumerary α-cells to transdifferentiate into cells with a β-cell phenotype may assist in development of strategies for replenishment of β cells. We bred the glucagon receptor mutant (*gcgr*$^{-/-}$) zebrafish with an α-cell reporter line *Tg(gcga:GFP)*, and then treated them with artemether, GABA and aspterric acid. After treatment, we stained these *gcgr*$^{-/-}$*; Tg(gcga:GFP)* zebrafish with insulin antibody, and analyzed their α and β cells. Artemether and aspterric acid significantly decreased the number of α cells (Fig. 5A and 5B). Strikingly, the bi-hormonal (glucagon- and insulin-co-expressing) endocrine cells significantly increased (Fig. 5A and 5C). Moreover, the expression level of *insa* was upregulated after treatment with artemether and aspterric acid (Fig. 5D-5E). However, the transcription from the glucagon genes did not change (Fig. 5F-5G), which may have been due to the high levels of expression of glucagon genes in *gcgr*$^{-/-}$ mutant zebrafish (Li, Dean, Zhao, Nicholson, Powers & Chen, 2015). Taken together, these data indicated that artemether and aspterric acid are able to convert the supernumerary α cells to β cells.

3.6 Artemether and aspterric acid stimulated α- to β-cell transdifferentiation by up-regulating the expression of Pax4

To explore the mechanism by which artemether and aspterric acid potentiated α-cell transdifferentiation, we assessed the expression of transcription factors which are necessary for maintaining the α- or β-cell identity by qRT-PCR. After treatment with artemether and aspterric acid, we found that in both αTC1-6 cells and islets isolated from zebrafish larvae, the expression level of *Pax4* was significantly upregulated (Fig. 6A-6F). In addition, *Ins2, Mafa* and *Ucn3*, markers of mature β cells, were also upregulated after artemether and aspterric acid

treatment (Fig. 6A-6F). However, expression profiles for other transcriptional factors, *Arx*, *Pdx-1* and *Nkx6.1*, *Nkx2.2, Pax6*, *Syt4* were not altered (Fig. 6A-6F). In the GABA-treated group, an increase in *Mafa* and *Ucn3* expression were found in αTC1-6 cells and isolated zebrafish islets (Fig. 6B and 6E).

To further confirm the results obtained from qRT-PCR, we used a dual-luciferase reporter assay to assess the expression level of *Pax4*. The mouse *Pax4* promoter was subcloned upstream of firefly luciferase gene in a PGL6 vector, and the renilla luciferase reporter gene plasmid was used as an internal reference. As shown in Fig. 6G, the relative luciferase activity was increased in the group treated with artemether and aspterric acid, but not GABA. These results suggested that artemether and aspterric acid induced α to β cell transdifferentiation through upregulation of *Pax4* expression (Fig. 6H).

4. DISCUSSION

The zebrafish is an ideal model for studying β-cell regeneration, including the α to β cell transdifferentiation, due to availability of powerful genetic and chemical tools (Yang, Covington & Chen, 2020; Ye, Robertson, Hesselson, Stainier & Anderson, 2015). In this study, we used these features of the zebrafish as a model and performed a small molecule screening with sesquiterpenoids and diterpenoids. Based on visibly fluorescent (both α and β cells were labeled) transgenic zebrafish, we demonstrated that artemether and aspterric acid induced pancreatic α cells to transdifferentiate into β cells *in vivo*.

Artemether increases GABA signaling and prevents α cell glucagon secretion, which in turn suppresses the α cell-fate determining transcription factor Arx, resulting in the α to β cell transdifferentiation (Li et al., 2017). At the same time, long-term GABA treatment induced precursor cell neogenesis, passing through a glucagon-expressing transitional phase, and then the conversion of α cells into functional β cells (Ben-Othman et al., 2017). These observations are significant to the diabetes field, since these two small molecular compounds provide

potential β cell restorative treatments for patients with diabetes. However, contrary to this, two later studies suggested that artemether and GABA did not induce α cell to β cell conversion *in vivo* or *in vitro* using mouse models (Ackermann, Moss & Kaestner, 2018; van der Meulen et al., 2018). Instead, they suggested that artemether impaired β cell characteristics and led to β cell dedifferentiation (van der Meulen et al., 2018). These differences may have been due to the different experimental conditions. For example, the conclusions of Li and colleagues were based on observations in the mouse and zebrafish *in vivo,* as well as αTC1-6 cells and human islets *in vitro* (Li et al., 2017). In contrast, the studies by van der Meulen and colleagues relied only on isolated mouse or human islets *in vitro* (van der Meulen et al., 2018). Moreover, other factors, such as mouse strain, diet and housing conditions, as well as the heterogeneity of human islets, may also have contributed to the discrepancy.

In our study, to clarify whether artemether could be useful in this important debate, we used several different assays to test α to β cell transdifferentiation. We firstly identified that the artemether and aspterric acid increased the percentage of bi-hormonal cells (positive for insulin and glucagon) in zebrafish, increasing insulin-producing β cells, while concurrently decreasing glucagon-producing α cells (Fig. 1). These data suggested that the artemether and aspterric acid induced α cell transdifferentiation into β cells in zebrafish. Secondly, using lineage tracing, we further demonstrated increased recombinant eGFP positive cells (Fig. 2A-2C). Our lineage tracing results indicated that it is theoretically possible that these phenomena were due to induction of glucagon in β cells, rather than because of insulin induction in α cells. However, this possibility was excluded by qRT-PCR analysis, which revealed that artemether and aspterric acid upregulated the expression of *ins* but not *gcg* or *arx* (Fig. 1G-1I, Fig. 6A-6F).

Thirdly, these newly formed β cells, induced by artemether and aspterric acid, were not from precursor cell differentiation or existing β cell proliferation, as there were no changes in the Neurod1/ Insulin or EdU/insulin double-positive cells (Fig 3). Our data in zebrafish were

consistent with reports by Li et al. (Li et al., 2017), although we used a shorter treatment period. Moreover, as the precursor marker Neurod1 positive cell did not change, and was accompanied by an α-cell decrease and β-cell increase (Fig. 3A, Fig. 1D and Fig. 1E), we infer that artemether and aspterric acid converted α cells directly into new β cells, rather than through dedifferentiation to an immature bi-hormonal state.

Similar to Li et al. (Li et al., 2017), we also induced zebrafish to develop a diabetes-like state using a inducible system to target the proapoptotic protein tBID, to induce apoptosis in the zebrafish β cells (Li, Maddison, Page-McCaw & Chen, 2014). Artemether and aspterric acid treatments significantly increased bi-hormonally positive cells as well as β cells, which were functional as shown by decreased free glucose level in the cultures compared to the control (Fig. 4). All these results in zebrafish were consistent with those of Li et al. (Li et al., 2017), although they used a different zebrafish β-cell ablation model (targeted induction of caspase-8 in the β cells). Furthermore, in an α-cell hyperplasia model, generated by deficiency of glucagon receptor, artemether and aspterric acid converted the supernumerary α cells into β cells (Fig.5). Taken together, these data confirmed that artemether and aspterric acid were able to induce α-cell transdifferentiation into β cells under the increased demand of β cell regeneration, or when hyperplasia of α cells was experimentally induced.

In the *in vitro* studies, we generated a stable Ins2-eGFP αTC1-6 cell line in which Artemether and aspterric acid significantly increased eGFP expression, suggesting increased insulin transcription (Fig. 2E and 2F). Furthermore, the β cell markers *MafA* and *Ucn3* (Fig. 6A and 6C) increased, which suggested conversion of the αTC1-6 cells into β-like cells, concurring with Li et al. (Li et al., 2017). However, *Arx* was neither suppressed by artemether nor aspterric acid (Fig. 6). Interestingly, the expression of the β-cell fate marker *Pax4* was also significantly increased by artemether and aspterric acid (Fig. 6A and 6C), together with an increase in *Pax4* promoter-derived luciferase construct expression (Fig. 6G). Further, using

isolated zebrafish islets, we showed elevated *pax4* expression in the islets following artemether and aspterric acid treatment. Taken together, our data suggested that artemether and aspterric acid induced the zebrafish α cell and αTC1-6 cell transdifferentiation into β cells, through induction of *Pax4* expression, a factor essential for pancreatic β cell/δ cell differentiation and maintenance of β cell identity. The importance of Pax4 has previously been inferred by Pax4 mutant (*pax4*$^{-/-}$) mice displayed complete loss of β and δ cells and died about 3 days after birth because of severe diabetes (Sosa-Pineda, Chowdhury, Torres, Oliver & Gruss, 1997). In contrast, ectopic expression of *Pax4* converted mouse α to β cells during development and when triggered in adulthood (Al-Hasani et al., 2013; Collombat et al., 2009). Furthermore, overexpression of *Pax4* stimulated phenotypic conversion of α to β cells in the αTC1-9 cell line (Courtney et al., 2013). All these results indicate a crucial role for Pax4 in α-cell transdifferentiation to β cells.

In our study, GABA increased the insulin-producing β cells and decreased glucagon-producing α cells in zebrafish (Fig. 1D and 1E). However, we suggest a different mechanism to that discussed for artemether and aspterric acid, as GABA appeared to induce precursor cell differentiation to β cells instead of α cell transdifferentiation, based on several lines of evidence. Firstly, GABA did not change the percentage of bi-hormonal cells (Fig. 1C). Secondly, the result of lineage tracing in zebrafish did not support increased β cell conversion from α cells when zebrafish were incubated with GABA (Fig. 2B and 2C). Thirdly, GABA did not increase GFP expression in the αTC1-6 cell line with Ins2-eGFP stable expression (Fig. 2E and 2F). Last but not least, GABA treatement increased NeuroD1- and Mnx1-positive β cells in zebrafish (Fig. 3A and 3C, and Supplemental Fig. 5). Our conclusion that GABA induces precursor cell differentiation is in part similar to Ben-Othman et al. (Ben-Othman et al., 2017); however, we only observed GABA-induced precursor cell neogenesis in zebrafish, while Ben-Othman et al., suggested that GABA induced α cell precursor cell neogenesis and then

conversion of α to β-like cells in mice (Ben-Othman et al., 2017). These differences may due to the species differences or other factors.

In summary, we have shown that artemether and aspterric acid induced zebrafish α cell and αTC1-6 cell transdifferentiation into β cells (Fig. 6H). These two compounds also converted α cells into β cells, when β cells were lost or when α cells were hyperplastic. This α to β cell conversion occurred through increase of *Pax4* expression. Although there is still debate about whether the artemether- and GABA-induced α-cell transdifferentiation occurs in mouse and human islets (Ackermann, Moss & Kaestner, 2018; Ben-Othman et al., 2017; Li et al., 2017; van der Meulen et al., 2018), our data from zebrafish and the αTC1-6 cell are in line with the zebrafish and *in vitro* data reported by Li et al. (Li et al., 2017). Furthermore, our data, together with those of Li et al. (Li et al., 2017), suggest that artemether and aspterric acid are able to induce α-cell transdifferentiation into β cells, at least in zebrafish and αTC1-6 cells. The gene expression profiles and responses may not be identical among zebrafish, mouse and human. Further work is clearly required in mice, and importantly in humans to ascertain whether this could be an important therapeutic strategy in the future.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Natural Products Research, Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Gelling RW, Du XQ, Dichmann DS, Romer J, Huang H, Cui L, *et al.* (2003). Lower blood glucose, hyperglucagonemia, and pancreatic alpha cell hyperplasia in glucagon receptor knockout mice. Proc Natl Acad Sci U S A 100: 1438-1443.

Harlan DM, Kenyon NS, Korsgren O, Roep BO, & Immunology of Diabetes S (2009). Current advances and travails in islet transplantation. Diabetes 58: 2175-2184.

Helker CSM, Mullapudi ST, Mueller LM, Preussner J, Tunaru S, Skog O, *et al.* (2019). A whole organism small molecule screen identifies novel regulators of pancreatic endocrine development. Development 146.

Kang MS, Hirai S, Goto T, Kuroyanagi K, Kim YI, Ohyama K, *et al.* (2009). Dehydroabietic acid, a diterpene, improves diabetes and hyperlipidemia in obese diabetic KK-Ay mice. Biofactors 35: 442-448.

Kang Q, Hu M, Jia J, Bai X, Liu C, Wu Z, *et al.* (2020). Global Transcriptomic Analysis of Zebrafish Glucagon Receptor Mutant Reveals Its Regulated Metabolic Network. Int J Mol Sci 21.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, & Schilling TF (1995). Stages of embryonic development of the zebrafish. Dev Dyn 203: 253-310.

Lee YS, Lee C, Choung JS, Jung HS, & Jun HS (2018). Glucagon-Like Peptide 1 Increases beta-Cell Regeneration by Promoting alpha- to beta-Cell Transdifferentiation. Diabetes 67: 2601-2614.

Li J, Casteels T, Frogne T, Ingvorsen C, Honore C, Courtney M, *et al.* (2017). Artemisinins Target GABAA Receptor Signaling and Impair alpha Cell Identity. Cell 168: 86-100 e115.

Li M, Dean ED, Zhao L, Nicholson WE, Powers AC, & Chen W (2015). Glucagon receptor inactivation leads to alpha-cell hyperplasia in zebrafish. The Journal of endocrinology 227: 93-103.

Li M, Maddison LA, Page-McCaw P, & Chen W (2014). Overnutrition induces beta-cell differentiation through prolonged activation of beta-cells in zebrafish larvae. Am J Physiol Endocrinol Metab 306: E799-807.

Li M, Page-McCaw P, & Chen W (2016). FGF1 Mediates Overnutrition-Induced Compensatory beta-Cell Differentiation. Diabetes 65: 96-109.

Liu SZ, Tang XX, He FM, Jia JX, Hu H, Xie BY, *et al.* (2020). Two new compounds from a mangrove sediment-derived fungus Penicillium polonicum H175. Nat Prod Res: 1-9.

Maddison LA, & Chen W (2012). Nutrient excess stimulates beta-cell neogenesis in zebrafish. Diabetes 61: 2517-2524.

Matsuoka TA, Kawashima S, Miyatsuka T, Sasaki S, Shimo N, Katakami N, *et al.* (2017). Mafa Enables

Pdx1 to Effectively Convert Pancreatic Islet Progenitors and Committed Islet alpha-Cells Into beta-Cells In Vivo. Diabetes 66: 1293-1300.

Migliorini A, Bader E, & Lickert H (2014). Islet cell plasticity and regeneration. Mol Metab 3: 268-274.

Nair GG, Tzanakakis ES, & Hebrok M (2020). Emerging routes to the generation of functional beta-cells for diabetes mellitus cell therapy. Nat Rev Endocrinol 16: 506-518.

Obholzer N, Wolfson S, Trapani JG, Mo W, Nechiporuk A, Busch-Nentwich E, *et al.* (2008). Vesicular glutamate transporter 3 is required for synaptic transmission in zebrafish hair cells. J Neurosci 28: 2110-2118.

Perez Gutierrez RM, & Baez EG (2020). Diterpenes from seeds of Phalaris canariensis and their PTP1B inhibitory activity and hypoglycemic effects in streptozotocin-induced diabetic mice. J Asian Nat Prod Res 22: 603-617.

Shahjalal HM, Abdal Dayem A, Lim KM, Jeon TI, & Cho SG (2018). Generation of pancreatic beta cells for treatment of diabetes: advances and challenges. Stem Cell Res Ther 9: 355.

Sosa-Pineda B, Chowdhury K, Torres M, Oliver G, & Gruss P (1997). The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. Nature 386: 399-402.

Thorel F, Nepote V, Avril I, Kohno K, Desgraz R, Chera S, *et al.* (2010). Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 464: 1149-1154.

Unger RH, & Cherrington AD (2012). Glucagonocentric restructuring of diabetes: a pathophysiologic and therapeutic makeover. J Clin Invest 122: 4-12.

van der Meulen T, Lee S, Noordeloos E, Donaldson CJ, Adams MW, Noguchi GM, *et al.* (2018). Artemether Does Not Turn alpha Cells into beta Cells. Cell Metab 27: 218-225 e214.

Wei R, Cui X, Feng J, Gu L, Lang S, Wei T, *et al.* (2020). Dapagliflozin promotes beta cell regeneration by inducing pancreatic endocrine cell phenotype conversion in type 2 diabetic mice. Metabolism 111: 154324.

Weir GC, Gaglia J, & Bonner-Weir S (2020). Inadequate beta-cell mass is essential for the pathogenesis of type 2 diabetes. Lancet Diabetes Endocrinol 8: 249-256.

Xie S, Sinha RA, Singh BK, Li GD, Han W, & Yen PM (2013). Resveratrol induces insulin gene expression in mouse pancreatic alpha-cells. Cell Biosci 3: 47.

Yang B, Covington BA, & Chen W (2020). In vivo generation and regeneration of beta cells in zebrafish. Cell Regen 9: 9.

Yang YP, Thorel F, Boyer DF, Herrera PL, & Wright CV (2011). Context-specific alpha- to-beta-cell reprogramming by forced Pdx1 expression. Genes Dev 25**:** 1680-1685.

Ye L, Robertson MA, Hesselson D, Stainier DY, & Anderson RM (2015). Glucagon is essential for alpha cell transdifferentiation and beta cell neogenesis. Development 142**:** 1407-1417.

Yu R (2018). Mahvash Disease: 10 Years After Discovery. Pancreas 47**:** 511-515.

Zecchin E, Filippi A, Biemar F, Tiso N, Pauls S, Ellertsdottir E*, et al.* (2007). Distinct delta and jagged genes control sequential segregation of pancreatic cell types from precursor pools in zebrafish. Developmental biology 301**:** 192-204.

Zhang Z, Hu Y, Xu N, Zhou W, Yang L, Chen R*, et al.* (2019). A New Way for Beta Cell Neogenesis: Transdifferentiation from Alpha Cells Induced by Glucagon-Like Peptide 1. J Diabetes Res 2019**:** 2583047.

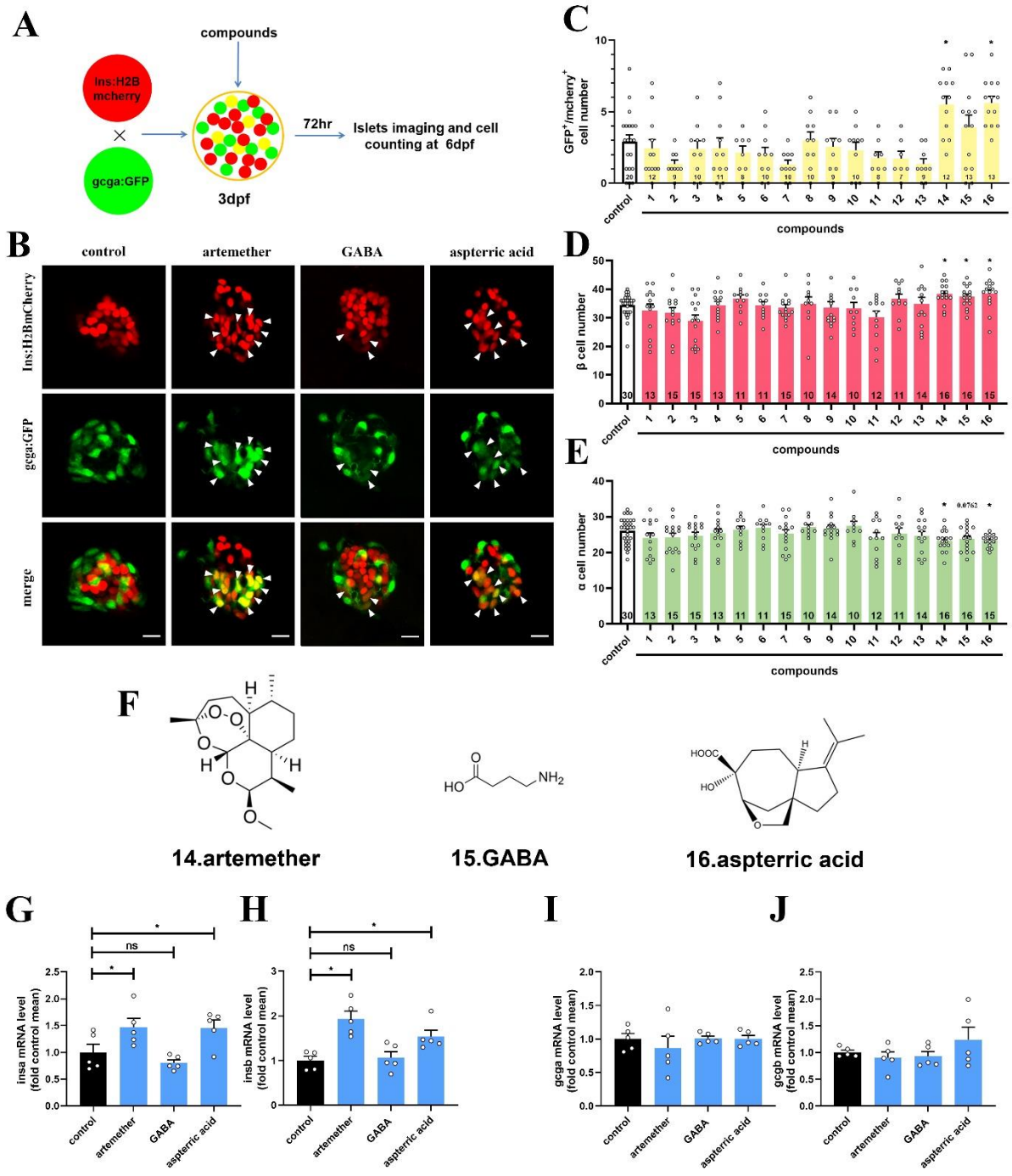

Figure 1. Small molecule screening identified two sesquiterpenes and GABA increased β-cell generation by α-cell transdifferentiation.

A. Schema of small molecule compounds screening (list shown in supplementary table 1) using transgenic zebrafish as the model. After treatment with 10μM compounds from 3 to 6 dpf (days post-fertilization), double transgenic zebrafish *Tg(gcga:GFP); Tg(Ins:H2BmCherry)* were fixed and imaged under confocal fluorescence microscopy and then endocrine cells were counted. **B.** Representative confocal projections of *Tg(gcga:GFP);Tg(Ins:H2BmCherry)* at 6

dpf after treatment with artemether, GABA and aspeterric acid. Cells which expressed both insulin ($mCherry^{+}$) and glucagon (GFP^{+}) are indicated by arrows. Scale bar indicates 10μm. **C.** Quantification of GFP and mCherry double positive cells in each group. **D-E**. Quantification of β cells (**D**) and α cells (**E**) in each group. Data are presented as Mean ± S.E.M; the number of zebrafish larvae in each group is shown on the bars of the graph. **F**. The structure of three candidate compounds is shown. **G-J**. qRT-PCR analysis of insulin (**G-H**) and glucagon (**I-J**) genes expression in compound-treated zebrafish larvae, 30 larvae were used for total RNA extraction in each group. The data presented here are Mean ± S.E.M of five independent experiments (n=5). *, $P < 0.05$ *vs* control; ns, no significant difference. Data in **1C, 1D, 1E and 1G** were analyzed by the parameter analysis using one-way ANOVA (F at $P < 0.05$) with Bonferroni's *post hoc* test; data in **1H** was analyzed by non-parametric Kruskal–Wallis test; data in **1I and1J** were analyzed by the parameter analysis with one-way ANOVA, but no post-hoc test (F at $P > 0.05$), there was no difference among these groups.

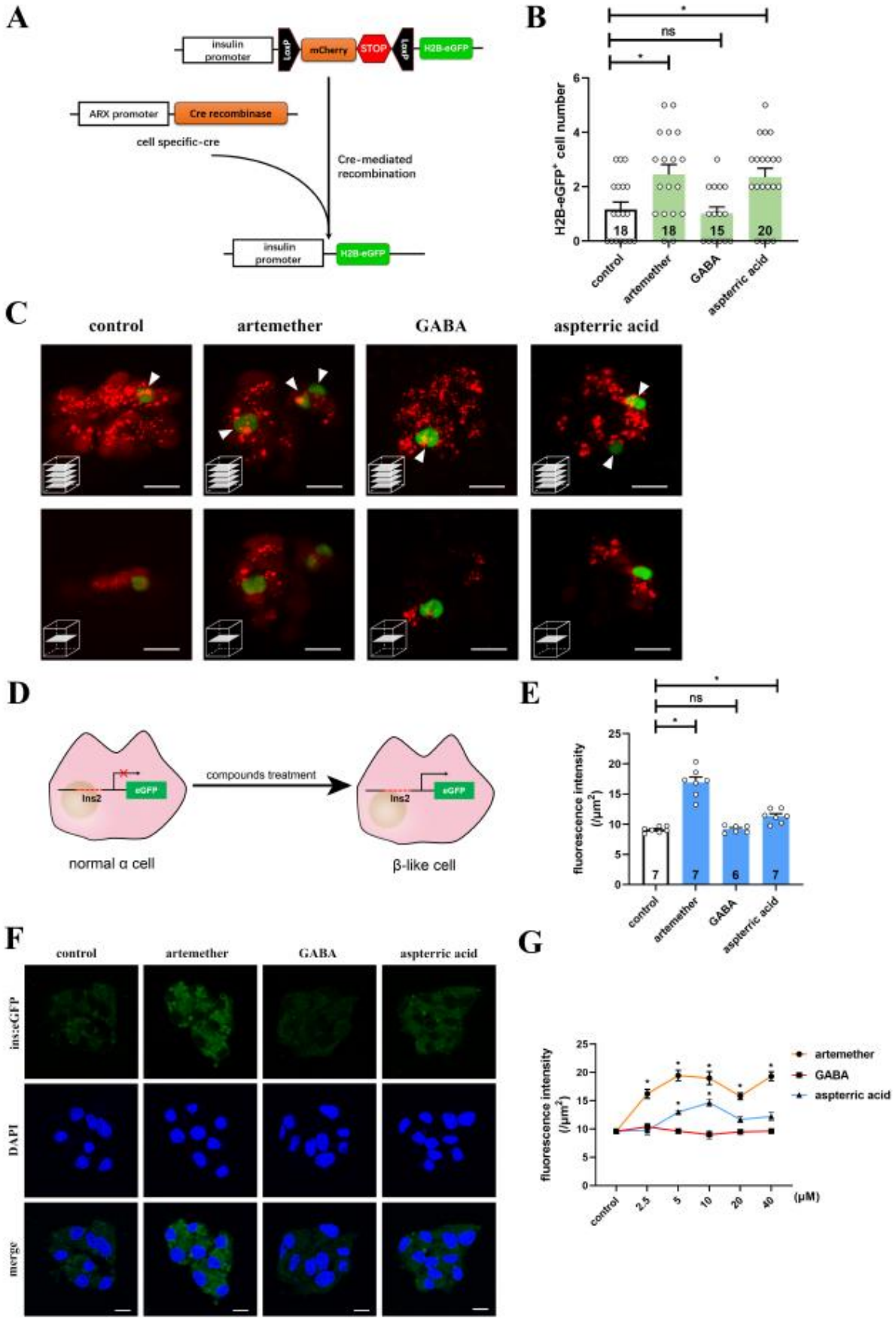
A
insulin promoter
LoxP
mCherry
STOP
LoxP
H2B-eGFP
ARX promoter
Cre recombinase
cell specific-cre
Cre-mediated recombination
insulin promoter
H2B-eGFP
B
ns
*
*
H2B-eGFP+ cell number
18
18
15
20
control
artemether
GABA
aspterric acid
C
control
artemether
GABA
aspterric acid
D
Ins2
eGFP
compounds treatment
Ins2
eGFP
normal α cell
β-like cell
E
ns
*
*
fluorescence intensity (/μm2)
7
7
6
7
control
artemether
GABA
aspterric acid
F
control
artemether
GABA
aspterric acid
ins:eGFP
DAPI
merge
G
fluorescence intensity (/μm2)
artemether
GABA
aspterric acid
control
2.5
5
10
20
40
(μM)

Figure 2. Artemether and aspterric acid induced α to β cell conversion by *in vivo* lineage tracing and *in vitro* assays

A-C. Lineage-tracing evidence supports α- to β-cell transdifferentiation after 72hr artemether and aspterric acid treatment. **A**. Scheme for *in vivo* α cell lineage tracing; Tg^{BAC}*(arx:Cre)* zebrafish line was crossed with the *Tg(ins:loxP:mCherrySTOP: loxP:H2B-eGFP)* reporter line. If α cells convert to β cells, the loxP-mediated excision of the STOP cassette permits these transdifferentiated cells to specifically express the stable fusion-protein H2B-eGFP. **B**. Insulin-expressing cells that originate from α cells, with the number of zebrafish larvae in each group shown on the bars of the graph. Data shown are means ± S.E.M. **C**. Representative confocal projections (upper panel) or single slices (lower panel) at 6 dpf, with H2B-eGFP cells indicated by arrows; scale bar indicates 10μm. **D-F.** Artemether and aspterric acid induced α-cell transdifferentiation *in vitro*. **D**. Representation of the plasmid vector used for establishing stable transfected cell lines, the eGFP reporter gene was under the control mouse insulin2 promoter, and once α cells convert into β cells, the insulin promoter initiates the expression of eGFP. **E**. Comparison of eGFP fluorescence intensity after the cells were treated with 10 μM of each compound for 72 hr. Data shown are means ± S.E.M (n=7). **F**. Representative confocal images of cells which were treated with 10 μM compounds for 72 hr. Scale bars indicate 10 μm. **G.** Measurement of the eGFP fluorescence intensity after the cells were treated with different concentrations of compounds for 72hr. *, $P < 0.05$ *vs* control; ns, no significant difference. Data in **2B** was analyzed by non-parametric Kruskal–Wallis test; Data in **2E** and **2G** were analyzed by the parameter analysis using one-way ANOVA (F at $P < 0.05$) with Bonferroni's *post hoc* test.

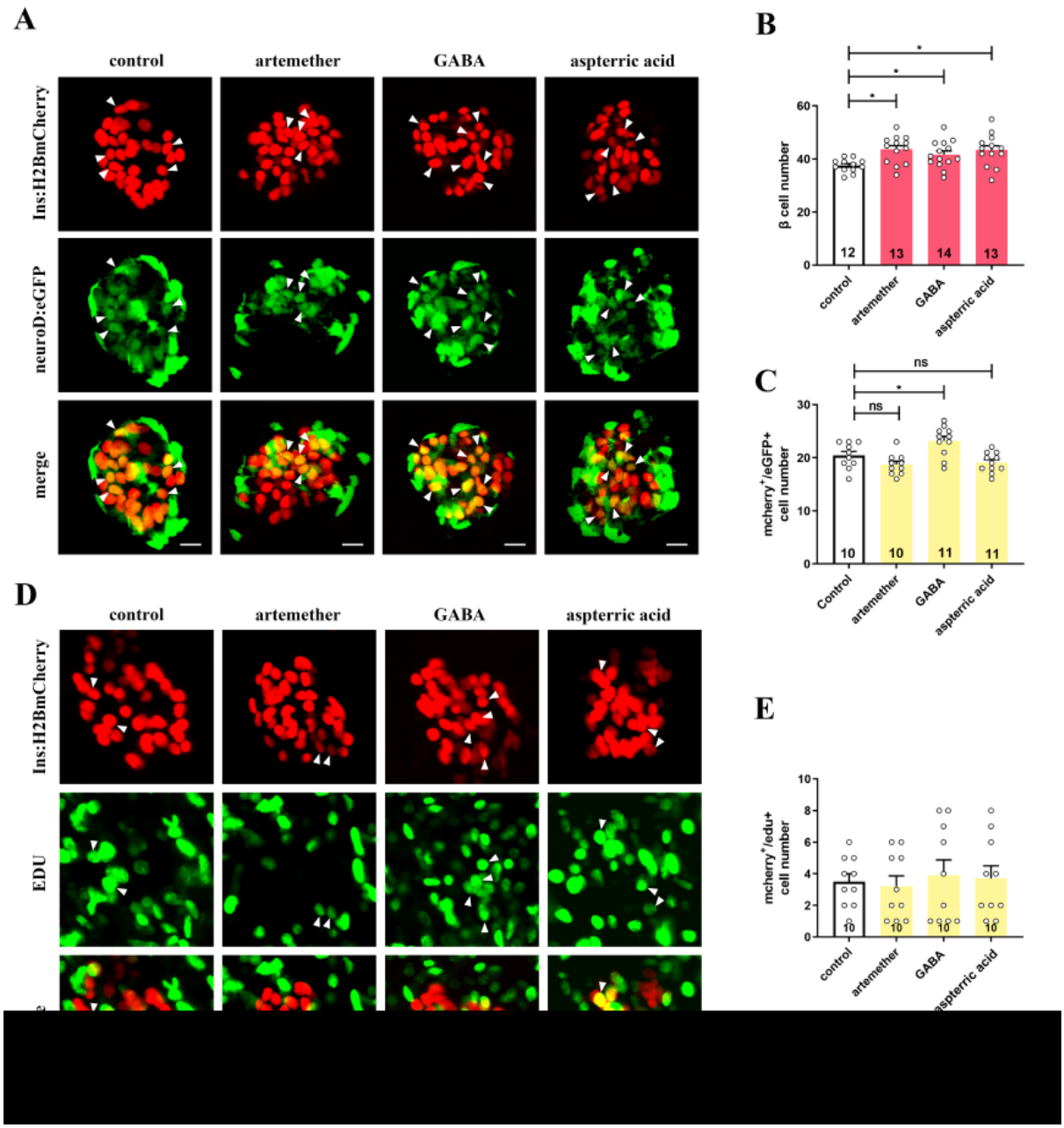

Figure 3. Artemether and aspterric acid neither induce differentiation of endocrine precursors nor induce replication of β-cell proliferation.

A-C. Determination of the effect of artemether, GABA and aspterric acid on the stimulation of precursor cell differentiation. **A**. Representative confocal projections of double transgenic zebrafish *Tg*^{BAC}*(Neurod1:eGFP);Tg(Ins:H2BmCherry)* at 6 dpf, after treatment with 10 μM of each compound for 72 hr, mCherry^{+}/eGFP^{+} cells are indicated by arrows. Scale bar indicates 10 μm. **B**.Quantification of the β cells. **C**. Quantification of mCherry^{+} and eGFP^{+} double positive cells. **D-E.** Determination of the effect of artemether, GABA and aspterric acid on the stimulation of β cell replication by EdU staining. Compound treatment of *Tg(ins:H2BmCherry)*

transgenic larvae was carried out as above with EdU added at 4dpf for 48hr. **D**. Representative confocal projections of *Tg(ins:H2BmCherry)* after staining with EdU; $mCherry^{+}/EdU^{+}$ cells are indicated by arrows. Scale bars indicate 10 μm. **E**. Quantification of $mCherry^{+}$ and EdU^{+} double positive cells. All the data shown here are the means ± S.E.M; the number of zebrafish larvae in each group is shown within the bars, *, $P < 0.05$ *vs* control; ns, no significant difference. Data in **3B** and **3C** were analyzed the parameter analysis using one-way ANOVA (F at $P < 0.05$) with Bonferroni's *post hoc* test; data in **3E** were analyzed by non-parametric Kruskal–Wallis test and there was no difference among these groups.

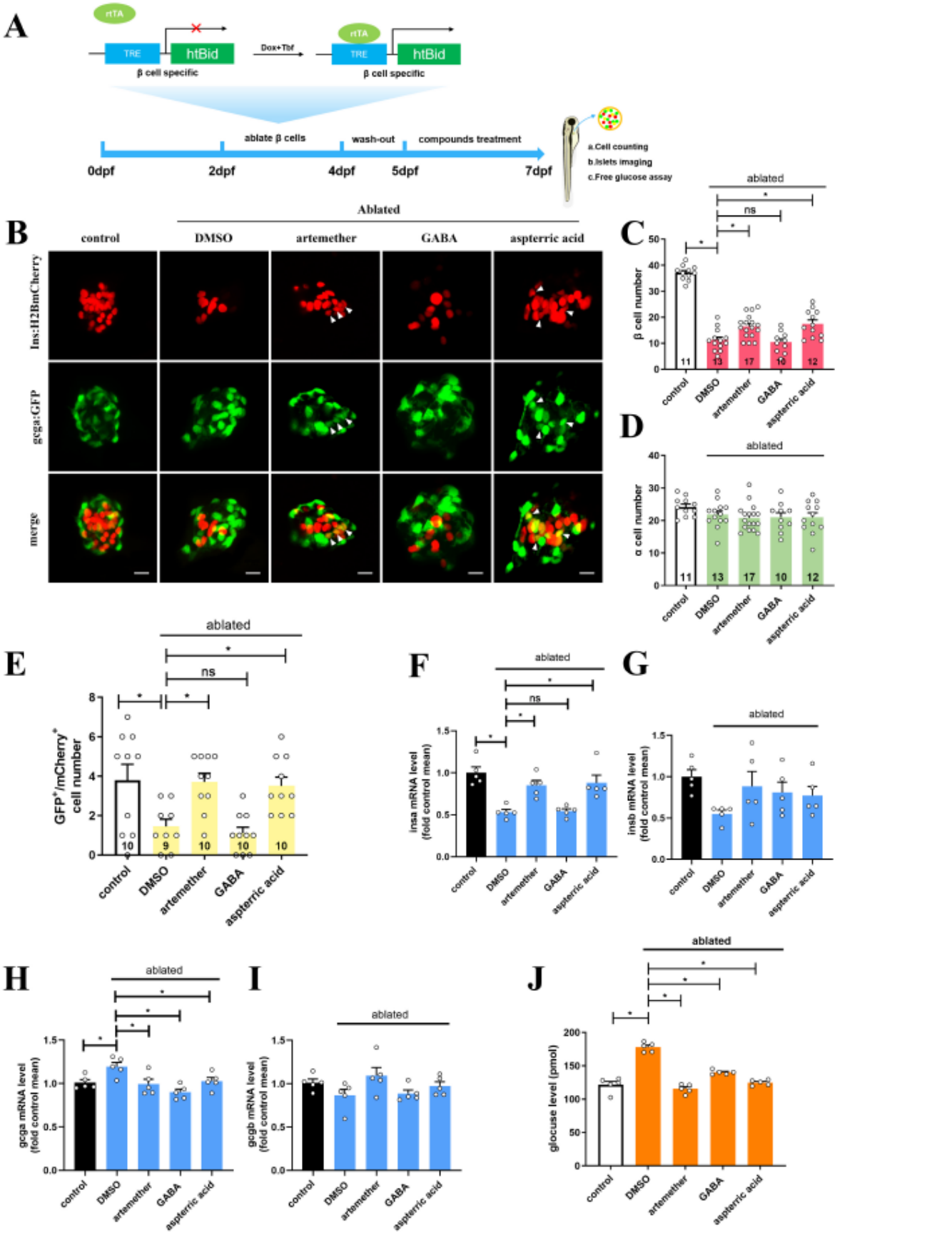
A
rtTA
TRE
htBid
β cell specific
Dox+Tbf
rtTA
TRE
htBid
β cell specific
ablate β cells
wash-out
compounds treatment
0dpf
2dpf
4dpf
5dpf
7dpf
a.Cell counting
b.Islets imaging
c.Free glucose assay
B
Ablated
control
DMSO
artemether
GABA
aspterric acid
Ins:H2BmCherry
gcga:GFP
merge
C
ablated
β cell number
control
DMSO
artemether
GABA
aspterric acid
D
ablated
α cell number
E
ablated
GFP+/mCherry+ cell number
F
ablated
insa mRNA level (fold control mean)
G
ablated
insb mRNA level (fold control mean)
H
ablated
gcga mRNA level (fold control mean)
I
ablated
gcgb mRNA level (fold control mean)
J
ablated
glocuse level (pmol)

Figure 4. Artemether and aspterric acid induce α to β cell conversion after β cell ablation in zebrafish

A. Schema of the procedures to ablate β cells followed by compound treatment. Triple transgenic zebrafish *Tg(Ins:htBid$^{TE\text{-}ON}$;LR);Tg(Ins:H2BmCherry);Tg(gcga:GFP)* were incubated with doxycycline and tebufenozide at 2 dpf for 48 hr to ablate their β cells and subsequently washed with 0.3X Danieau solution for 24hr, then compound treatment was started at 5 dpf for 48 hr, with a concentration of 10μM. The control group was raised in the same conditions as the experimental groups, but these larvae did not have β cells ablated. **B**. Representative confocal projections of triple transgenic larvae at 7 dpf after incubation with different compounds. Cells which co-expressed insulin (mCherry^{+}) and glucagon (GFP^{+}) are indicated by arrows, and the scale bar indicates 10 μm. **C-D**. Quantification of β cells (**C**) and α cells (**D**) in each group separately. **E**. Quantification of bi-hormonal (mCherry^{+}/GFP^{+}) cells. Data presented in (**C**-**E**) are Mean ± S.E.M, with the number of zebrafish larvae in each group was shown in the bars. **F-I**. qRT-PCR analysis of insulin and glucagon gene expression in β-cell ablated zebrafish larvae, and compound treatments were carried out as described in (A), 30 larvae were collected in each group used for total RNA extraction. The data presented here are Mean ± S.E.M of five independent experiments (n=5). **J**. Glucose measurement in pooled larvae extracts, with compound treatments carried out as in (A) and the data presented here are Mean ± S.E.M of five replicates (n=5). *, $P < 0.05$ *vs* DMSO; ns, no significant difference. Data in **4C**, **4E**, **4F**, **4H** and **4J** were analyzed by the parameter analysis using one-way ANOVA (F at $P < 0.05$) with Bonferroni's *post hoc* test.; data in **4D** and **4I** was analyzed by the parameter analysis with one-way ANOVA, but no post-hoc test (F at $P > 0.05$), there was no difference among these groups.; data in **4G** was analyzed by non-parametric Kruskal–Wallis test and there was no difference among the groups.

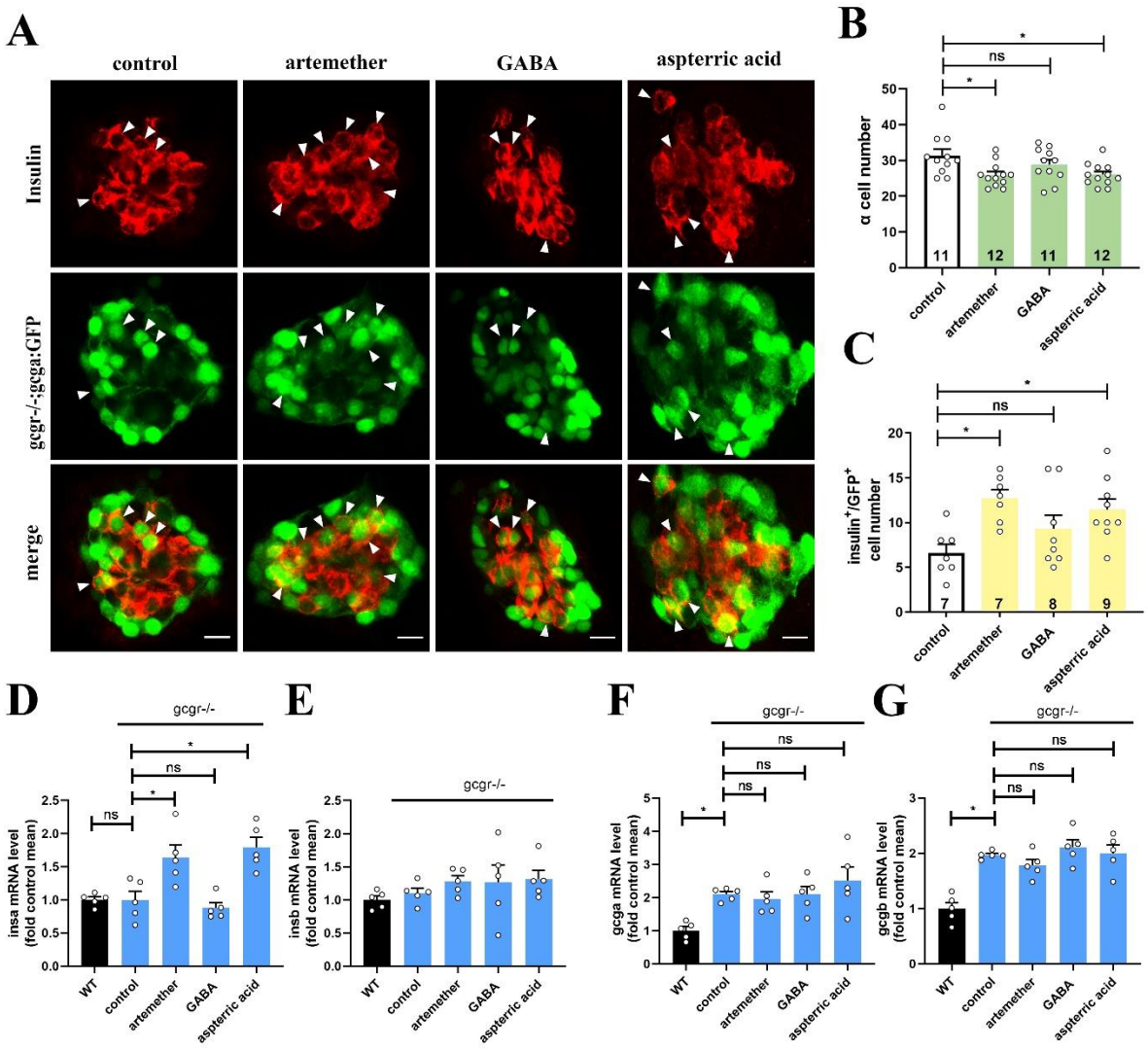

Figure 5. Artemether and aspterric acid induced α cells to transdifferentiate to β cells in an α-cell hyperplasia model

A. Representative confocal projections of *gcgr*$^{-/-}$*;Tg(gcga:GFP)* zebrafish at 6 dpf after treatment with different compounds at 10 μM; the control group was treated with equal volume of DMSO. Whole-mount immunofluorescence staining was used to detect the expression of insulin with anti-insulin antibody. Cells which co-express insulin and glucagon (GFP^{+}) are indicated by arrows, and the scale bar indicates 10 μm. **B.** Quantification of α cells. **C.** Quantification of bi-hormonal (insulin^{+}/GFP^{+}) cells. Data presented in (B&C) are Mean ± S.E.M, with the number of zebrafish larvae in each group shown within the bars. **D-G**. qRT-PCR analysis of insulin and glucagon genes expression in *gcgr*$^{-/-}$*;Tg(gcga:GFP)* zebrafish at 6 dpf after treatment with the different compounds at 10 μM; 30 larvae were collected in each group for total RNA extraction. The data presented here are Mean ± S.E.M of five independent

experiments (n=5). *, $P < 0.05$ *vs* control; ns, no significant difference. Data in **5B**, **5C**, **5D**, **5F** and **5G** were analyzed by the parameter analysis using one-way ANOVA (F at $P < 0.05$) with Bonferroni's *post hoc* test; data in **5E** were analyzed by the parameter analysis with one-way ANOVA, but no post-hoc test (F at $P > 0.05$), there was no difference among these groups.

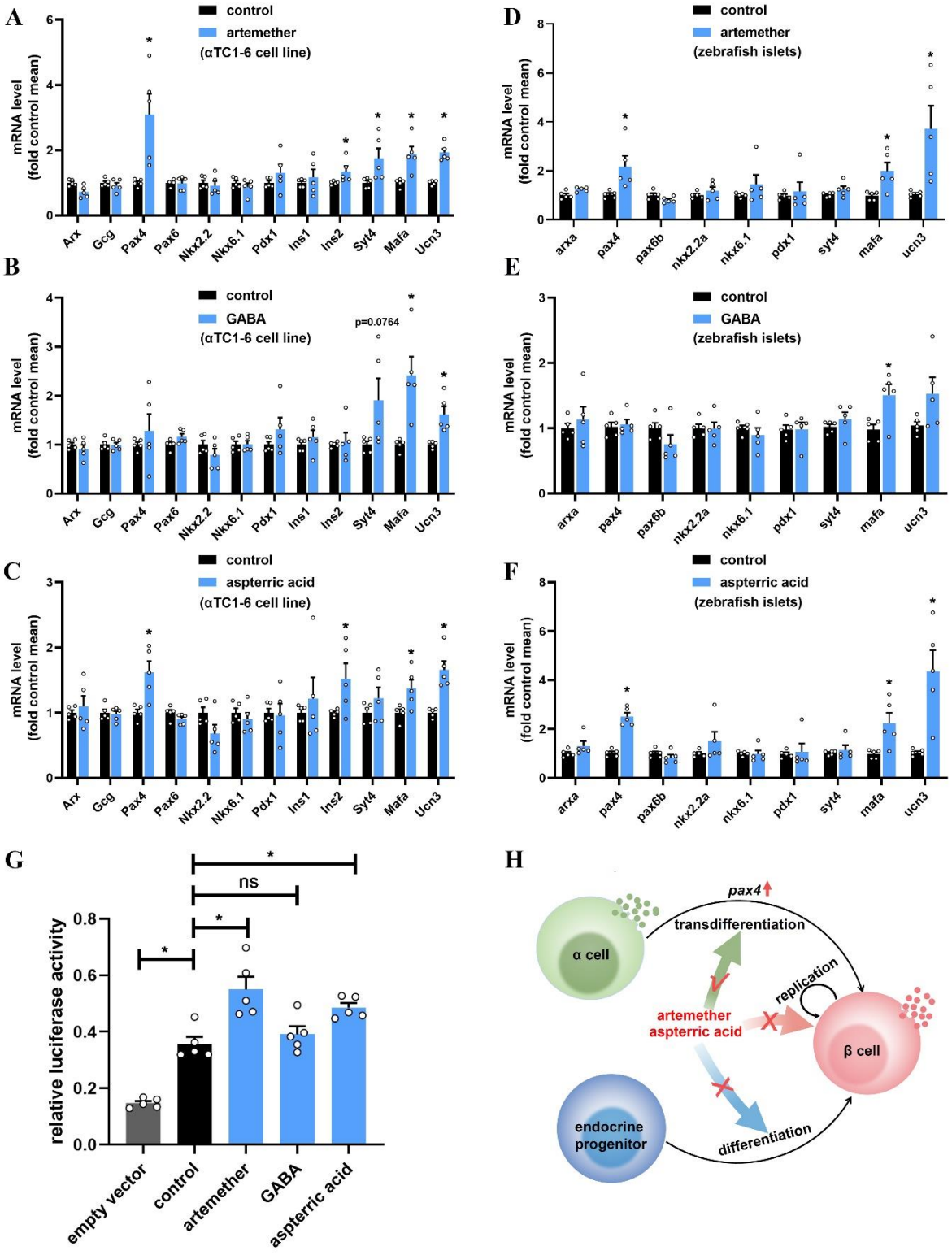

Figure 6. The expression level of *Pax4* was upregulated by artemether and aspterric acid

A-F. qRT-PCR analysis of relative genes expressed in the αTC1-6 cell line and isolated zebrafish islets after treatment with the different compounds. **A-C**. The αTC1-6 cell line was treated with 10 μM artemether (**A**), GABA (**B**) or aspterric acid (**C**) for 72 hr and extracted

total RNA was used for analysis of gene expression. **D-F**. Zebrafish larvae islets were isolated after the larvae were treated with 10 μM artemether (**D**), GABA (**E**) or aspterric acid (**F**) for 72 hr and extracted total RNA was used to analyze the relative gene expression, compared with the control. Data presented in (**A-F**) are Mean fold change ± S.E.M of 5 independent experiments (n=5). **G.** Luciferase assay to show the expression of *Pax4* after the αTC1-6 cell line was treated with 10μM compounds for 72 hr. The data presented here are Mean ± S.E.M of five independent experiments (n=5). **H.** Working model for artemether and aspterric acid induced α cell to β cell conversion. *, $P < 0.05$ *vs* control; ns, no significant difference. Data in **6A-6F** were analyzed by unpaired t test; data in **6G** were analyzed by the parameter analysis using one-way ANOVA (F at $P < 0.05$) with Bonferroni's *post hoc* test.