

Chemical screen identifies FDA-approved drugs and target pathways that induce precocious pancreatic endocrine differentiation

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Pancreatic β -cells are an essential source of insulin and their destruction because of autoimmunity causes type 1 diabetes. We conducted a chemical screen to identify compounds that would induce the differentiation of insulin-producing β -cells in vivo. To do this screen, we brought together the use of transgenic zebrafish as a model of β -cell differentiation, a unique multiwell plate that allows easy visualization of lateral views of swimming larval fish and a library of clinical drugs. We identified six hits that can induce precocious differentiation of secondary islets in larval zebrafish. Three of these six hits were known drugs with a considerable background of published data on mechanism of action. Using pharmacological approaches, we have identified and characterized two unique pathways in β -cell differentiation in the zebrafish, including down-regulation of GTP production and retinoic acid biosynthesis.

progenitor | Notch-signaling | development | embryogenesis

Type 1 diabetes in humans and nonobese diabetic (NOD) mice is caused by a T-cell-dependent destruction of pancreatic β -cells. Elimination of the β -cells abrogates production of insulin, a hormone required for regulating blood glucose levels. One approach to recovering glucose homeostasis would be to induce endogenous regeneration of β -cells. There is evidence for the potential of mammalian β -cell regeneration. In long standing type 1 diabetes in humans, persistent and/or regenerating β -cells remain scattered in the pancreas, along with continued apoptosis of β -cells (1). Similarly, β -cell mass can be restored to cure type 1 diabetes in NOD mice when treated with immunosuppressors (2). These results suggest a capacity for regeneration of endogenous β -cells in diabetic patients. Therefore, identification of drugs that stimulate β -cell production will be useful in providing therapeutic solutions for diabetes.

As with mammals, the β -cells of the zebrafish are located in the endocrine islets of the pancreas. In the zebrafish, there are two waves of endocrine formation during early development. By day one of embryogenesis, insulin expressing cells can be seen as a principal islet, a large anterior accumulation of endocrine cells (3). A second wave of β -cell differentiation (or secondary transition) starts 80 h after fertilization (hpf) because endocrine cells differentiate from the extrapancreatic duct and contribute to the principal islet (4). Starting ≈ 5 d after fertilization (dpf), secondary transition continues as progenitors residing within the intrapancreatic duct differentiate to form small accumulations of endocrine cells known as the secondary islets (5, 6). Importantly, only endocrine cells formed during these later events (secondary transition) have proliferative potential and give rise to the majority of the adult endocrine pancreas (7). Precocious formation of secondary islets can be induced before 5 dpf by addition of the Notch inhibitor, DAPT (6). In this manner, zebrafish β -cell differentiation is analogous to the process in mammals, involving ductal-associated progenitors under the control of the Notch-signaling pathway.

Our goal was to find compounds that can induce secondary islet formation without embryo-wide loss of essential Notch signaling. The main impediment in moving a “hit” to clinical drug is the level of safety testing that such lead compounds must undergo. One way to circumvent this hurdle is to screen drugs already approved for clinical use (8). Any hit from such a library, irrespective of their intended use, can greatly reduce the lag between finding a candidate drug and going to clinical trials in humans. To expedite the eventual application of hit compounds to patients, we have assembled the Johns Hopkins Drug Library (JHDL), which consists of mainly clinically approved drugs (9, 10). The JHDL can be considered to be a collection enriched for biologically active compounds. We developed a zebrafish screening assay using multiwell plates that permit visualization of pancreata in living larvae. Using this assay, we screened the JHDL and identified hits that induced precocious β -cell differentiation. These hits included three FDA-approved drugs. Characterization of the hits led to the identification of the GTP and retinoic acid biogenesis pathways as critical pathways in β -cell differentiation.

Results

Chemical Screen for Inducers of β -Cell Differentiation. To screen numerous drugs on embryonic samples we developed SideView plate technology. Because of the construction of these plates (Fig. 1 A–C), a lateral view of larvae including the pancreas is permitted (Fig. 1 D and E). We have shown that pancreatic Notch-responsive cells (PNCs) are progenitors with potential to differentiate into the hormone producing cells of the endocrine pancreas (5). Inhibiting Notch-signaling induces precocious endocrine formation in the tail of the pancreas concomitant with loss of PNCs in the developing duct (6). Although Notch inhibition induces differentiation of β -cells (6), it is an unlikely therapeutic strategy for diabetes, because Notch has multiple functions throughout the body. We carried out a screen for compounds that could induce secondary islets without a widespread change in the Notch-signaling pathway. To do this screen, we used fish transgenic for two constructs: (i) *Tp1:hmgb1-mCherry*(6), where a Notch-responsive element drives expression of nuclear-mCherry; and (ii) *pax6b:GFP*, where the endocrine precursors and hormone producing cells are marked with GFP

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Conflict of interest statement: A.A.F. is a full-time employee of Physical Sciences, Inc., which makes, distributes, and sells the SideView microplate used in paper.

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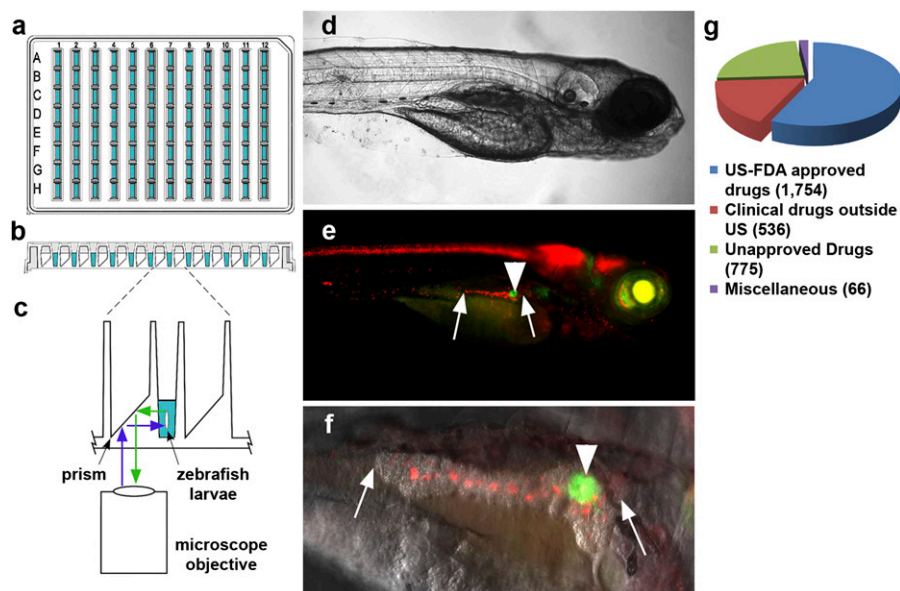


Fig. 1. Components of the chemical screen. (A) SideView plates contain 96 rectangular wells with same external dimensions and spacing as standard 96-well plate. (B) Plate cross-section. (C) Adjacent prism refracts both the excitation light and fluorescence emission (blue and green lines, respectively); this set-up allows lateral visualization of living fish and pancreas. (D and E) Images of free-swimming, 5 dpf larvae in SideView plate (5 $\times$), oriented to show anterior to the right and dorsal up. Transgenics for *Tp1:hmgbl-mCherry* and *pax6b:GFP* brightfield image (D) and merged fluorescent image (E). (F) High magnification (20 $\times$) of mounted larvae. (E and F) The posterior (left white arrow) and anterior (right white arrow) limits of the pancreas and the location of the endocrine cells of the principal islet are indicated (white arrowhead). (G) Chart representing the 3,131 compounds in the JHDL.

(11). Using double transgenic fish (Fig. 1 E and F), we could observe both Notch signaling and early endocrine differentiation.

Unlike the cells of the principal islet, the larval secondary islets are formed solely by differentiation from ductal progenitors. This process is equivalent to mammalian secondary transition where the mature β -cells are first formed. For this reason, we focus our screen on isolating chemical inducers of precocious secondary islets. To maximize our chances of finding compounds that induce endocrine differentiation, we used the JHDL (Fig. 1G). Two double-transgenic embryos at 2.5 dpf were placed into each well. Each chemical from the JHDL was added into a single well to give a final concentration of 20 μ M. By 5 dpf, the larvae were examined by fluorescence microscopy for appearance of secondary islets. During development, secondary islets only rarely form before 6 d of development (6). With the condition of this screen and at the level of detection used (inverted compound scope), no secondary islets were detected in 672 DMSO control larvae at 5 dpf. Of the 3,131 drugs tested, 8.6% were lethal, and 89.5% had no effect on secondary islet formation; of the remaining drugs, 18 had an effect on precocious secondary islet formation in both larvae tested (Fig. S1). Further experiments with fresh stock solution of these 18 drugs identified six hit compounds with consistent results: Tetraethylthiuram disulfide [Disulfiram, DSF; Chemical Abstracts Service (CAS) no. 97-77-8], mycophenolic acid (MPA; CAS no. 24280-93-1), levallorphan tartrate (CAS no. 152-02-3), Esculin monohydrate (CAS no. 531-75-9), Epirizole (Mepirizole; CAS no. 18694-40-1), and Sulfanilate Zinc (sulfanilic acid; CAS no. 515-74-2).

FDA-Approved DSF and MPA Induce Precocious Secondary Islet Formation. As proof of principle for this screen, we decided to focus on elucidating the mechanism of DSF and MPA. Both drugs are FDA approved, DSF is prescribed for the treatment of alcohol abuse, and MPA is used as an immunosuppressant. Fig. 2 shows confocal images from pancreata microdissected from 6 dpf *pax6b:GFP*(11);*Tp1:hmgbl-mCherry*(6) double transgenic larvae. These larvae were treated from 3 dpf with DMSO alone, 100 μ M

DAPT, 10 μ M MPA, or 10 μ M DSF. As was seen in the screen, incubation with either MPA (Fig. 2C) or DSF (Fig. 2D) lead to precocious secondary islet formation as detected by the expression of *pax6b:GFP*. Similarly, we could detect precocious secondary

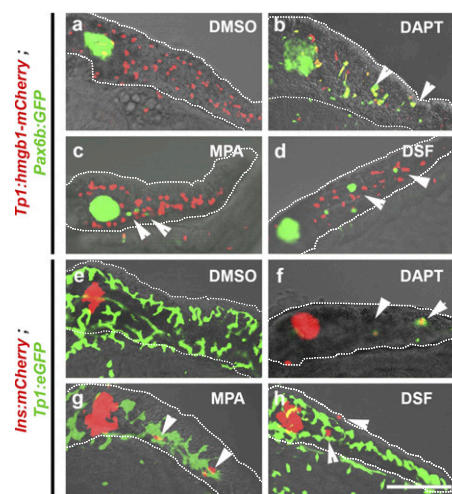


Fig. 2. Induction of precocious β -cell differentiation. Confocal images of microdissected pancreata (6 dpf) are oriented so the head of the pancreas (anterior) is on the left. (A–D) *Tp1:hmgbl-mCherry;pax6b:GFP* transgenics. Notch-responsive cells are red, and nascent endocrine cells are green. (E–H) *Tp1:eGFP;ins:mCherry* transgenics. Notch-responsive cells are green and β -cells are red. (A and E) Incubation with DMSO (0.5%) (3–6 dpf) led to no induction. (B and F) Notch-repression by 100 μ M DAPT (3–6 dpf) caused (B) endocrine differentiation including (F) β -cells throughout the pancreas. In both transgenic models, induction of endocrine cells by DAPT is accompanied by a reduction in Notch-responsivity. (C, D, G, and H) In contrast, the compounds MPA (10 μ M) and DSF (10 μ M) cause precocious secondary islets without affecting Notch-responsivity. White arrowheads indicate examples of secondary islets and pancreata are outlined by white dashed lines. (Scale bar: 100 μ m.)

majority of larvae treated with either MPA alone (76%) or MPA plus adenosine (50 μ M 67%, 100 μ M 68%) displayed induced secondary islets (Fig. 3 D and G); however, when guanosine was supplemented the proportion (50 μ M 31%, 100 μ M 45%) of larvae displaying induced secondary islets was significantly reduced (Fig. 3 E and G). By counting the numbers of secondary islet cells under the same treatment conditions, we also showed that guanosine significantly reduces the number of secondary islet cells induced by MPA (Fig. S4). Altogether, these data suggest that MPA is inducing precocious secondary islets through inhibition of IMPDH and a suppression of cellular GTP levels.

DSF Induces Precocious Secondary Islet Differentiation by Inhibiting Retinoic Acid Synthesis. DSF has several biologically activities. First, DSF is a known inhibitor of two very different enzymes: aldehyde dehydrogenase (ALDH) (14), and DNA methyltransferase-1 (DNMT-1) (15). Second, DSF is a chelator of heavy metals and was identified in a chemical screen in zebrafish as perturbing pigmentation, a copper-dependent process (16). As an ALDH inhibitor, DSF blocks the conversion of retinaldehyde to the morphogen retinoic acid (RA). Hence, DSF has been used in embryological studies to study the role of RA in development (17). It has been shown that RA signaling specifies the pancreatic field within the developing endoderm (18).

To test whether inhibiting RA production can recapitulate the induction of secondary islets seen with DSF, we assayed the activity of diethylaminobenzaldehyde (DEAB), another well-characterized inhibitor of RA synthesis (19). *Pax6:GFP;ins:mCherry* embryos were incubated from 3 dpf in DMSO alone (Fig. 4A), 10 μ M DSF (Fig. 4B), or 10 μ M DEAB (Fig. 4C). At 6 dpf, 20 pancreata were dissected and secondary islet cells were quantified. On average, 3.25 secondary islet cells per pancreas

were detected after DSF incubation and 2.8 cells for DEAB. Directly blocking the action of RA using the antagonist BMS493 (CAS no. 215030-90-3) also induced precocious differentiation of secondary islets (Fig. S5C). Furthermore, the action of BMS493 (Fig. S5D), DSF (Fig. S5A), and DEAB (Fig. S5B) can all be blocked by 10 μ M RA. Altogether, these data demonstrate that inhibiting RA production or function, between 3 and 6 dpf, causes precocious secondary islet formation in larval zebrafish.

Inhibiting both RA and Notch signaling induces early secondary islet formation, suggesting both pathways are involved in maintaining undifferentiated pancreatic progenitors. To investigate the relationship further, we asked whether RA could block the actions of Notch inhibition. Low doses of DAPT (10 μ M) induce significant numbers of secondary islets (Fig. 4D). RA (10 μ M) had no effect on secondary islet induction (Fig. 4E) and, when combined with DAPT, RA could block the action of Notch inhibition (Fig. 4F and G). Similar results were also obtained at higher doses (100 μ M) of RA and DAPT. By repeating these treatments with larvae transgenic for our Notch-responsive reporter line (*Tp1:hmgbl-mCherry*), we showed that Notch-signaling is inhibited by DAPT even in the presence of RA (Fig. 4K). These observations are consistent with DAPT blocking Notch-signaling in the progenitors, and complete differentiation to endocrine cells being blocked by RA. In other words, these data puts the role of RA downstream from Notch signaling in the endocrine differentiation pathway. Using a fluorogenic ALDH1 substrate (Aldefluor), we can detect ALDH enzymatic activity in the larval exocrine tissue (Fig. S5 E–J). This result strongly suggests there is an endogenous source of RA in the larval pancreas and supports the idea that RA and Notch are both involved in progenitor maintenance.

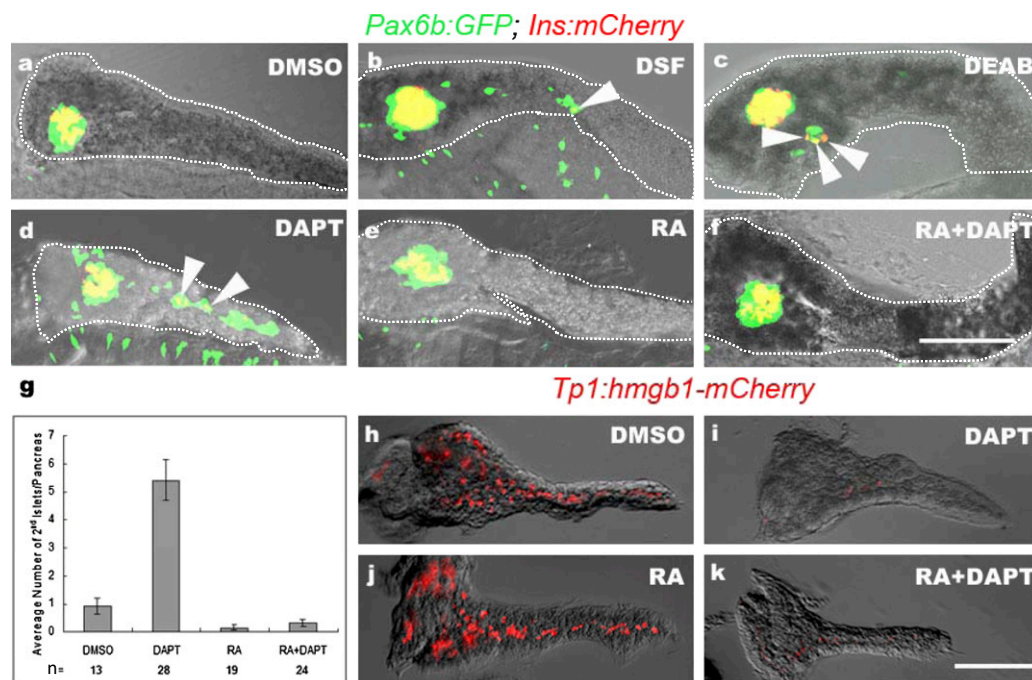


Fig. 4. The role of RA inhibition in secondary islet induction. (A–F) Confocal images of microdissected pancreata from *pax6b:GFP;ins:mCherry* larvae treated with (3–6 dpf): DMSO alone (A), 10 μ M DSF (B), 10 μ M DEAB (C), 10 μ M DAPT (D), 10 μ M RA (E), or 10 μ M RA and 10 μ M DAPT (F). The pancreata are outlined by a white dashed line. Secondary islets are induced by inhibitors of RA synthesis (B and C) and Notch signaling (D). Islets include β -cells (red fluorescence, arrowheads). Action of DAPT can be blocked by 10 μ M RA (F). The effects of RA on DAPT-dependent secondary islet induction was quantified and is represented in chart (G); the capacity of induction is shown as the average number of secondary islet cells per pancreas (y axis). Number of pancreata (n) analyzed for each condition is included below x axis. Bars represent the average result and error bars show SEM. (H–K) Pancreata from larvae carrying the Notch-responsive transgene, *Tp1:hmgbl-mCherry*, were treated with the same drugs and concentrations as above. DAPT inhibits Notch responsiveness whether incubated alone (I) or with RA (K). (Scale bars: 100 μ m.) All images show the anterior head of the pancreas to the left.

MPA, DEAB, and DAPT Induce β -Cell Differentiation in PANC-1 Cell Line. To test the effects of our hit compounds on human cells, we used PANC-1 cells, a line originally derived from a pancreatic duct carcinoma (20). PANC-1 cells can be induced to differentiate into hormone-producing islet-like clusters (21). We established the half maximal inhibitory concentration (IC_{50}) for PANC-1 cells incubated with DAPT (Fig. S6A), DSF (Fig. S6E and F), MPA (alone, with adenosine or guanosine; Fig. S6D), DEAB (Fig. S6B), and RA (Fig. S6C). DAPT, DEAB, and RA did not affect the proliferation of PANC-1 cells up to 50 μ M treatment. MPA, however, inhibited the cell proliferation with an IC_{50} value of 0.85 μ M. Guanosine, but not adenosine, could completely rescue the inhibition of PANC-1 cell proliferation by MPA, demonstrating an essential role of IMPDH in de novo synthesis of GTP and the growth of this cell line (Fig. S6D). DSF was inhibitory to PANC-1 cell proliferation over a wide range of concentrations, as it is to other cancer cell lines (22). Using an established protocol that makes PANC-1 permissive to endocrine differentiation (21), we tested the capacity of MPA (1 μ M), DEAB (1 μ M), DAPT (1 μ M), and RA (1 μ M) to induce the expression of insulin. Insulin transcription was ascertained by quantitative RT-PCR (qPCR). Compared with DMSO alone, DAPT, DEAB, and MPA treatment induced a 5.7-, 4.1-, and 4.8-fold increase of insulin transcripts, respectively (Fig. 5A). We also tested whether guanosine (20 μ M) could block the induction of insulin transcripts observed with MPA. The induction of insulin transcripts by MPA (4.8-fold increase) in PANC-1 cells was significantly reduced by guanosine (1.8-fold increase), suggesting that in vitro MPA also induces endocrine differentiation through the suppression of cellular GTP levels. As seen in larval zebrafish, in vitro RA can also block the effect of DAPT (Fig. 5A). Immunofluorescent staining demonstrated a corresponding increase in the number of insulin-positive cells. DAPT, DEAB,

and MPA treatment induced 2.75-, 1.85-, and 2.11-fold more insulin-positive cells than DMSO alone (Fig. 5B–F). This cell-based method allowed us to validate the effect of the hit, MPA, in a mammalian system and to further elucidate the roles of RA and Notch signaling in β -cell differentiation.

Discussion

Our studies presented here join the growing number of successful chemical screens undertaken in the zebrafish (23). We have carried out a screen for compounds that induce β -cell differentiation and identified both drug inducers and interesting pathways involved in β -cell differentiation. By compiling a drug library and developing a unique screening platform, we were able to identify multiple validated hits in a relatively small-scale, moderate-throughput screen. This finding has great implication for other workers in the field because high-throughput screens can be both costly and labor intensive.

MPA is a drug that inhibits the rate-limiting step in the de novo production of cellular GTP. As of yet, it is unclear why reduction of GTP would lead to precocious β -cell differentiation although IMPDH inhibition also has been shown to cause differentiation in a wide array of cancer cell lines and may be having a similar effect on β -cell progenitors (24). The effect of MPA on insulin secretion has been studied by several groups that showed MPA treatment leads to a reduction in β -cell hormone secretion (25, 26). Future work should be aimed at ascertaining the mechanism behind how GTP levels are involved in β -cell differentiation.

RA is a well-known morphogen used in multiple events during embryogenesis, including the specification of the zebrafish pancreas (18). In our screen, inhibiting RA production did not lead to fewer insulin-producing cells; indeed, inhibition of RA signaling through DSF induced differentiation of secondary islets

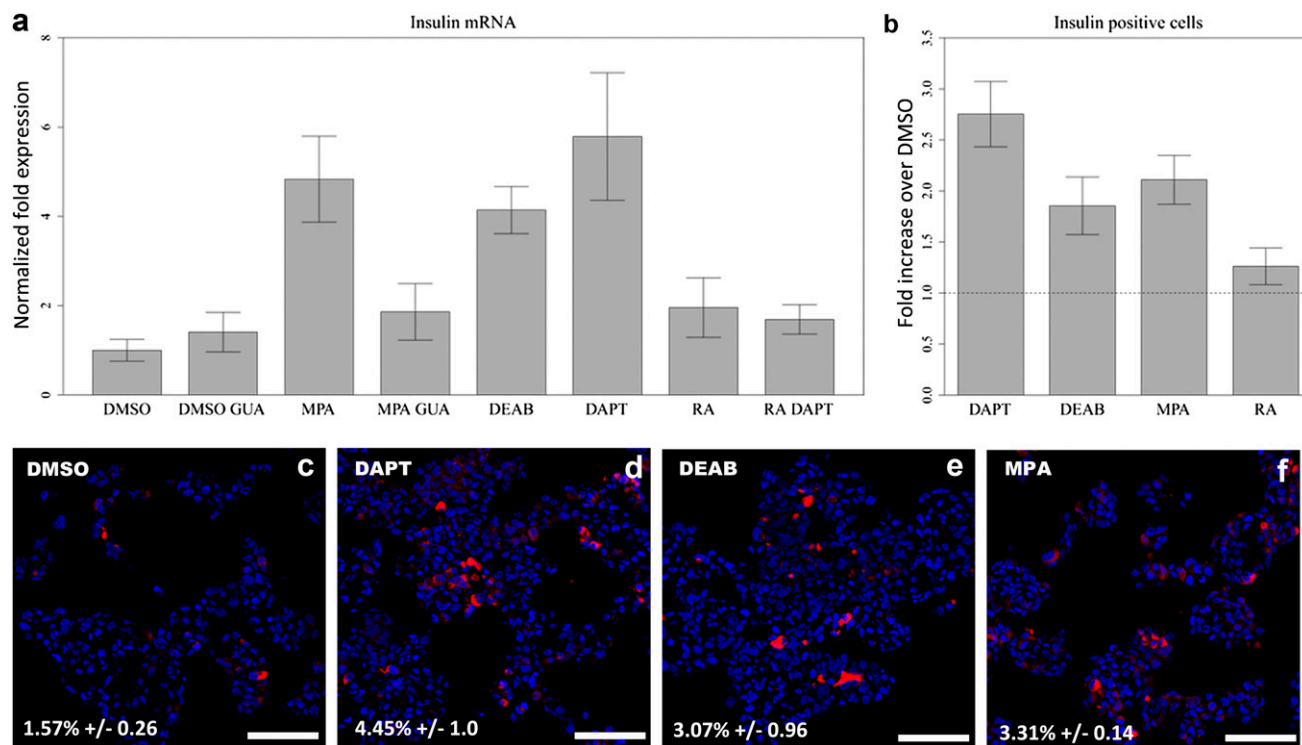


Fig. 5. Inhibiting the synthesis of Notch, GTP, or RA promotes the differentiation of PANC-1 cells to insulin-expressing cells. (A) Detection of the expression of insulin transcripts by qPCR after drug treatment. (B) Fold increase (over DMSO control) in the number of insulin-positive cells after treatment with DAPT, DEAB, MPA, and RA. DMSO level was set to 1 and represented by dashed line. (C–F) Immunofluorescence staining using anti-insulin antibody (red); nuclear staining with DAPI (blue). (Scale bars: 100 μ M.) Error bars represent SEM of three independent experiments.

β -cells. Prior work in zebrafish has demonstrated that RA plays a critical role in specifying pancreatic progenitors along the anterior-posterior axis (18). Presumably, because the pancreas is already specified in the late larval stages (2.5–5 dpf) used in the screen, all of the progenitors of the later forming secondary islets are already in place (6). Hence, this screen has identified a unique role for RA in pancreatogenesis. Our hypothesis is that RA is initially required for specification of the pancreas and is required later in development to maintain progenitors in an undifferentiated state.

PNCs are larval progenitors that reside in the developing pancreatic ducts and ultimately differentiate to adult cell types including endocrine cells (5). RA synthesis is catalyzed by the enzyme ALDH, and the exocrine cells surrounding the PNCs display ALDH activity (Fig. S5). Hence, there is a RA source in close proximity to larval pancreatic progenitors. This observation leads us to a hypothesis where a paracrine signal (RA) from one larval pancreas cell type is involved in maintaining the undifferentiated state of neighboring Notch-responsive pancreatic progenitors. Later in development, PNCs also differentiate to form the centroacinar cells (CACs) of the adult pancreas, a cell type that has also been implicated as having progenitor characteristics. At least some of these adult CACs also possess high ALDH activity (27). This concordance leads to the intriguing hypothesis that a similar relationship between RA and progenitors still exists in the adult pancreas tissue.

Materials and Methods

Transgenic Lines. Larvae for screening were generated by in-crossing *Tp1:hmgbl-mCherry^{h11}*; *pax6b:GFP^{ulg515}* double transgenics, where Notch-responsive cells are marked with red fluorescence (6) and nascent endocrine cells (and some neural tissues) with green (11). Other lines used are as follows: *ins:mCherry^{h2}* marking β -cells (6), *Tp1:eGFP^{um14}* marking Notch-responsive cells (6), *gcga:GFP^{ia1}* marking α -cells (28), *kdr1:GRCFP²ⁿ¹* marking blood vasculature (29), and *SST2:eGFP^{h20}* marking δ -cells.

Drug Library. The JHDL (9) was setup as follows: Each drug was made to 10 mM stock solutions with DMSO. The stock solutions were arrayed in a total

42 96-well plates, leaving the first and the last columns in each plate as DMSO controls. Each solution in these master plates were diluted (1× PBS) to make 200 μ M predilution plates (stored at -20°C).

Medium-Throughput Screening in 96-Well Plates. Transparent *Tp1:hmgbl-mCherry*; *pax6b:GFP* transgenic larvae were generated by incubating embryos from 1 dpf in 0.003% 1-phenyl 2-thiourea (PTU). At 2.5 dpf, 2 larvae per well were transferred into 96-well SideView plates containing E3 embryo medium with 0.003% PTU and 0.3% DMSO. Ten microliters of each compound from the predilution plate was added to larvae in the screening plate. The final volume per well was adjusted to 100 μ L with E3 to give 20 μ M final concentration. The concentration of vehicle was equivalent to that added to the drug treatment wells. Plates were sealed (Breathe-Easy; Sigma-Aldrich) and incubated (28°C) in dark until 5 dpf, then examined by inverted microscope, Axiovert 200 M (Zeiss).

Compounds that induced secondary islets in both larvae were investigated further. Where possible, new dilutions of stock solution were used and added to at least four SideView wells containing two embryos each. From their results each compound was characterized as follows: “Weak hit”—secondary islets in <40% of larvae. Hit—secondary islets in >80% of larvae. “False positive” did not induce secondary islets. “Candidate Hit”—stock solution unavailable, but did induce secondary islets in >80% of larvae using more aliquots from predilution plate.

For all other work, compounds were purchased (Sigma), made into 10 mM stock (in DMSO) and diluted to: 10 μ M MPA (M3536), 10 μ M DSF (86720), 10 μ M DEAB (D86256), 10 μ M Ribavirin (RV; R9644), 100 μ M Mizoribine (MZ; M3047), 10 μ M All-trans retinoic acid (RA; R2625) 10 μ M BMS493 (B6688), and 100 μ M *N*-[*N*-(3, 5-Difluorophenacetyl)-*L*-alanyl]-5-phenylglycine *t*-butyl ester (DAPT; D5942). Unless stated, 3dpf embryos were incubated in drug until 5dpf in the dark at 28°C .

For more information on confocal imaging, cell culture, immunofluorescent staining, and qPCR, see *SI Materials and Methods*.

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