



Transient FOXO1 inhibition in pancreatic endoderm promotes the generation of NGN3+ endocrine precursors from human iPSCs

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ABSTRACT

In the multi-step differentiation protocol used to generate pancreatic endocrine cells from human pluripotent stem cells, the induction of NGN3+ endocrine precursors from the PDX1+/NKX6.1+ pancreatic endoderm is crucial for efficient endocrine cell production. Here, we demonstrate that transient, not prolonged FOXO1 inhibition results in enhanced NGN3+ endocrine precursors and hormone-producing cell production. FOXO1 inhibition does not directly induce NGN3 expression but stimulates PDX1+/NKX6.1+ cell proliferation. NOTCH activity, whose suppression is important for Ngn3 expression, is not suppressed but Wnt signaling is stimulated by FOXO1 inhibition. Reversely, Wnt inhibition suppresses the effects of FOXO1 inhibitor. These findings indicate that FOXO1 and Wnt are involved in regulating the proliferation of PDX1+/NKX6.1+ pancreatic endoderm that gives rise to NGN3+ endocrine precursors.

1. Introduction

In vitro generation of pancreatic endocrine cells from human pluripotent stem cells has attracted interest, not only for application to diabetes therapy but also to understand the mechanism underlying human pancreatic development (Rezania et al., 2013; Rezania et al., 2014; Pagliuca et al., 2014). The basic concept is to mimic the multi-step differentiation observed in murine pancreatic organogenesis. In mice, all pancreatic endocrine cells, including insulin-producing beta cells, glucagon-producing alpha cells, somatostatin-expressing delta cells, and pancreatic polypeptide-producing PP cells, are derived from Ngn3+ endocrine precursors (Gu et al., 2002). No endocrine cells form in Ngn3 null mice (Gradwohl et al., 2000), nor are they generated from NGN3-null human ES cells (McGrath et al., 2015), demonstrating the indispensable role of Ngn3 in pancreatic endocrine development. Considering that organ cell number is determined by the number of precursors in the murine pancreas (Stanger et al., 2007) and that Ngn3+ cells (Desgraz and Herrera, 2009; Miyatsuka et al., 2011) and early beta cells (Qiu et al., 2017) are less-proliferative, the generation of more NGN3+ precursors would favor more efficient production of pancreatic endocrine cells from human pluripotent stem cells.

In mice, fate determination into endocrine-, duct-, and exocrine lineages is spatiotemporally determined within the developing ductal branch structure of the pancreatic epithelium (Bastidas-Ponce et al.,

2017). Within the pancreatic buds that evaginate from the gut tube, epithelial cells form the ductal plexus around embryonic day 15.5 (E15.5) and are gradually remodeled into a branched ductal structure around E17.5. While exocrine acinar cells differentiate from the tip region of the ductal tree, Ngn3+ endocrine precursors leave the epithelial cord of Nkx6.1+ Pdx1+ trunk region. During this process, Ngn3 expression determines cell behavior. Cells with Ngn3 expression above the threshold value are allowed to leave the ducts and proceed along the endocrine differentiation pathway, while Ngn3-low cells adapt to the ductal fate (Wang et al., 2010; Bechard et al., 2016; Sharon et al., 2019a). Thus, the Ngn3 regulatory machinery is important for endocrine vs. ductal fate determination. However, there is limited information regarding murine pancreatogenesis. Inactivation of Hes1, the major Notch signaling effector, causes increased Ngn3+ cell production as Hes1 directly binds to the regulatory region of the *Ngn3* locus and inhibits transcription (Lee et al., 2001; Ahnfelt-Ronne et al., 2012). Furthermore, hypoxia induced factor 1α (Hif1α) depletion indirectly upregulates Ngn3 expression through Hes1 expression inhibition (Heinis et al., 2010). Understanding of this mechanism was eventually used to induce NGN3+ cells from human pluripotent stem cells via γ-secretase inhibitor treatment (Pagliuca et al., 2014) or high-oxygen culture (Rezania et al., 2014).

Increasing evidence suggests that forkhead box protein O1 (FoxO1) is involved in Ngn3 regulation. FoxO1 expression is detected at E14.5 in

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the pancreatic epithelium (Kitamura et al., 2009). Cre-mediated FoxO1 inactivation in Pdx1-Cre or Ngn3-Cre strains, which arises during the pancreatic and endocrine precursor stages, respectively, results in elevated Ngn3 expression (Talchai and Accili, 2015). Whether Ngn3 transcription is activated or proliferation of Nkx6.1+/Pdx1+ progenitors and/or Ngn3+ cells is stimulated by FoxO1 depletion is unknown. However, an increased Ngn3+ cell number is postulated because FoxO1-null mutant mice show huge islet formation.

In this study, we used the FOXO1 inhibitor, AS1842856 (Nagashima et al., 2010), to induce pancreatic endocrine cells from human pluripotent stem cells. Further, we demonstrate that transient, not prolonged, FOXO1 inhibition stimulates PDX1+/NKX6.1+ progenitor proliferation resulting in enhanced NGN3+ endocrine precursors and hormone-producing cell production. Through FOXO1 inhibition, Wnt signaling was activated, and the suppression of Wnt signaling inhibited the effects of AS1842856. These findings provide mechanistic insights into the developmental machinery in human pancreatic endocrine cells.

2. Materials and methods

2.1. Human iPSC growth and differentiation protocol

Human iPSCs (585A1, 604B1 (Okita et al., 2013), and NGN3 reporter cells derived from 604B1) were grown on feeder layers of mitomycin C-treated SNL cells in Primate ES Cell Medium (Reprocell) with 500 U/ml penicillin/streptomycin (Thermo Fisher Scientific) and 4 ng/ml recombinant human basic fibroblast growth factor (bFGF; Wako). For passaging, hiPSC colonies were dissociated by CTK solution (Suemori et al., 2006) and split between 1:4 and 1:10. Experiments with hiPSCs were approved by the ethics committee of the Department of Medicine and Graduate School of Medicine, Kyoto University.

The contents of differentiation media at each step are shown in Supplemental Table 1. The incubator was supplied with 5% CO₂ and 20% O₂, except for Stage 3 and 4 (5% CO₂ and 5% O₂).

Stage 1: We removed feeder cells with CTK solution and plated monolayers of single pluripotent cells using Accutase (Innovative Cell Technologies) on 12-well plates (Corning) pre-coated with 1:30 diluted Matrigel (Corning, No.354234). 5.0×10^5 single cells per well were treated with S1D0 (Stage 1 Day 0) media for 1 day. The cells were cultured with S1D1 media for 1 day, and then with S1D2 media for 2 days.

Stage 2 and 3: The supplements were changed to S2 media for 4 days. At S2D4, cells were dissociated into single cells with Accutase, reseeded into 60 mm dishes (Iwaki), and grown in S3 media for 3 days.

Stage 4: The medium was changed to S4D0 media. On S4D1, differentiated cells were scraped out and pipetted into cell clumps and suspended into ultra-low attachment 6-well plates with S4D1 media for 1 day. Then, the organoids were cultured in S4D2 media for 4 days. On S4D5, DMSO or 1 μ M AS1842856 (FoxO1 inhibitor, Calbiochem) was added into the medium for 24 hours.

Stage 5 and 6: The organoids were rinsed with DMEM and treated with S5 media for 3 days, followed by S6D0 media for 2 days, and finally maintained in S6D2 media, which was replaced every 5 days.

2.2. Immunocytochemistry and immunohistochemistry

2D-plated cells were fixed with 4 % paraformaldehyde (PFA) for 20 min, incubated for 30 min with 2.5% horse serum (Vector Laboratories), and incubated with primary antibodies (Supplemental Table 2) overnight at 4 °C. 3D-organoids were embedded in iPGel (GenoStaff), and transplanted kidney grafts were fixed with 4 % PFA overnight at 4 °C. For paraffin sections, the fixed samples were dehydrated in graded alcohol, immersed in Histo-Clear (National Diagnostics), and finally embedded into paraffin. Paraffin sections were cut into 3 μ m-thick slices. After deparaffinization and hydration, an antigen

retrieval procedure was performed with Target Retrieval Solution pH 6.0 (Dako). The sections were blocked for 30 min at room temperature with 2.5 % horse serum and then incubated with primary antibodies (Supplemental Table 2) overnight at 4 °C. 2D-plated cells or paraffin-embedded sections were washed in PBS and incubated for 1 h with secondary antibodies (Supplemental Table 2). Images were visualized using an HS All-in-one Fluorescence Microscope (BZ-9000E, Keyence).

2.3. Real-time PCR analysis

RNA was extracted from cells using an RNeasy micro kit (Qiagen). cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo). For real-time PCR analysis, mRNA expression was quantified by StepOne (Thermo Fisher Scientific) using FastStart Universal SYBR Green Master with Rox (Roche). Target gene expression was normalized to β 2-Microglobulin, and the relative expression is shown compared with the DMSO control except in Figs. 2F and P, S2A, S2B, and S4E. All primers used for real-time PCR are listed in Supplemental Table 3.

2.4. Wnt activation/inhibition experiments and mouse organ culture

To activate or inhibit Wnt signaling, 5 μ M CHIR99021 or 20 μ M XAV939 with 500 nM PKF118-310 was added to the S4D5 organoid or mouse pancreatic rudiment for 24 h. For mouse pancreatic explant culture, dorsal pancreatic buds at E11.5 were dissected as previously described (Greggio et al., 2014). Dissected tissue was cultured at the air-medium interface using Millicell culture plate inserts (Merck Millipore) with DMEM/F12 medium supplemented with 10% FBS (fetal bovine serum, Biosera) and 500 U/ml penicillin/streptomycin. Cells were grown at 37 °C in humidified 5% CO₂.

2.5. Establishment of the NGN3 reporter using the CRISPR/Cas9 system

The gRNA (GAGGGAGCCGGGAGCCGTAGAGG) was designed using CRISPRdirect (<https://crispr.dbcls.jp/>) (Naito et al., 2015) and was cloned into the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector. Genomic DNA was isolated from hiPSCs (604B1). Homologous DNA 882 bp upstream and 914 bp downstream of the target site was amplified from genomic DNA by PCR. Using an In-Fusion kit (Clontech), two homologous DNA sequences, a self-cleaving 2A peptide with EGFP, and a geneticin-selection cassette were subcloned into the linearized pENTR-Donor-MCS2.

Target iPSCs (604B1) were pretreated with 10 μ M Y-27623 for 1 h. The cells were washed with PBS and treated with CTK solution for 1 minute at 37 °C to remove feeder cells and washed again with PBS. Next, the iPSCs were further dissociated into single cells by Accutase treatment for 5 min at 37 °C. 10 μ g of pX330-U6-Chimeric_BB-CBh-hSpCas9 and 10 μ g of pENTR-Donor-MCS2 were electroporated into 1.0×10^6 cells using a NEPA 21 electroporator (poring pulse: pulse voltage, 125 V; pulse width, 5 ms; pulse number, 2; Nepagene).

Cells were plated onto one well of a six-well plate with feeder cells and treated with 10 μ M Y-27632 for 2 days. Geneticin (100 μ g/mL; Gibco) selection was applied after iPSC colonies were recovered. The geneticin-resistant colonies were dissociated into single cells, and 1000 cells were plated into a 10 cm dish with feeders. Each subclone was screened by genomic PCR (P1-P2 primer pairs, amplifying a fragment from upstream of the 3' arm to P2A, P3-P4 primer pairs, amplifying a fragment from NeoR to downstream of the 5' arm, and P1-P4 primer pairs) (Fig. S4A and B, and Supplemental Table 4). We selected clone #19, which expressed the self-cleaving 2A peptide with EGFP and the geneticin-selection cassette homozygously inserted to the NGN3 locus. The heterozygous OSR1-GFP reporter cell line and DLX5 were used as references (Mae et al., 2013) (Fig. S4C and Supplemental Table 4). The geneticin-selection cassette was excised by electroporating 10 μ g of pCXW-Cre-puro using a NEPA 21 electroporator. Excision was confirmed by PCR screening using P4-P5 primer pairs (Fig. S4D). All DNA

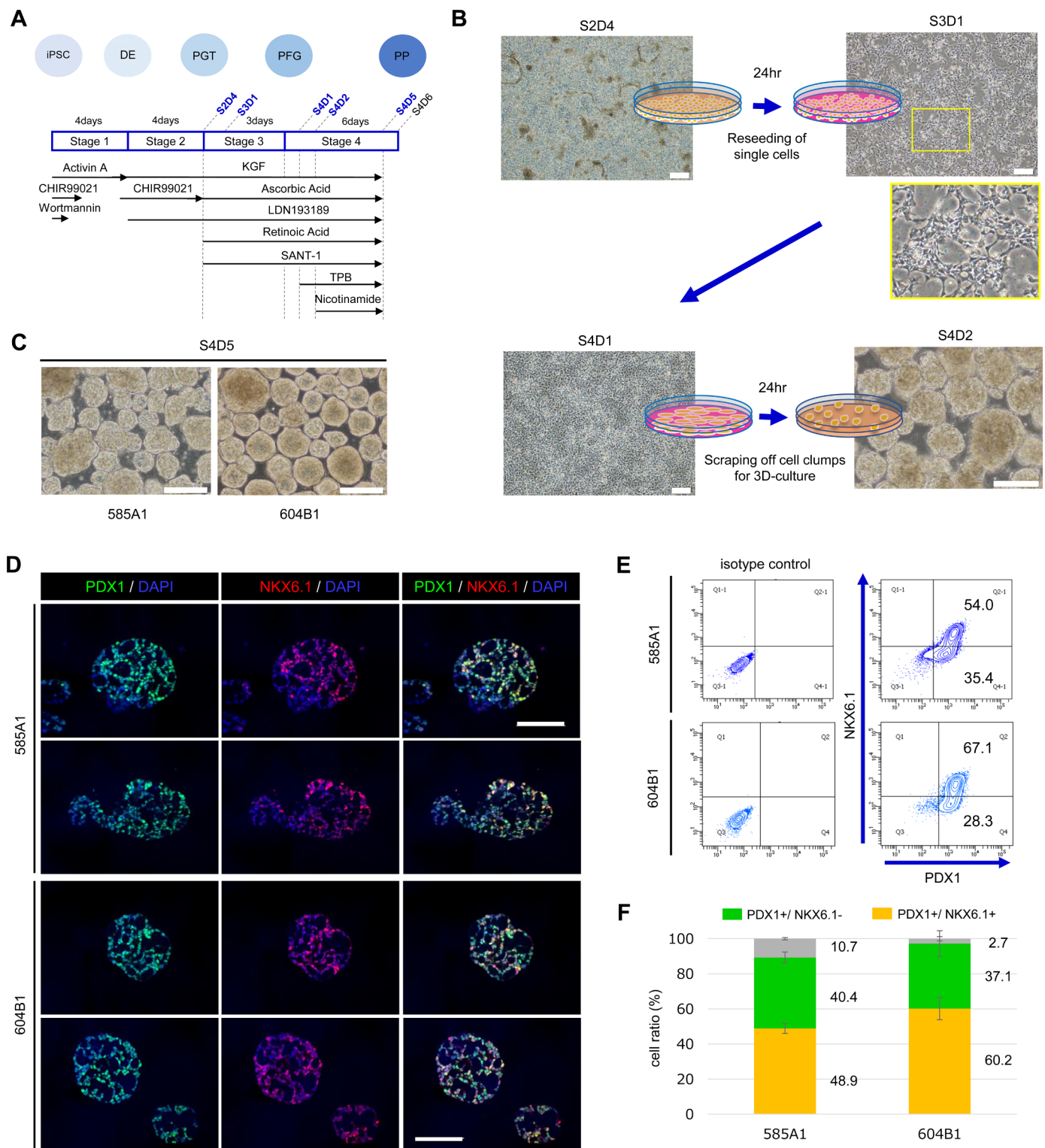


Fig. 1. Generation of PDX1 + /NKX6.1 + pancreatic organoids. (A) Stepwise protocol for pancreatic organoid generation from human iPSCs from Stage 1 to Stage 4. iPSC; induced pluripotent stem cells, DE; definitive endoderm, PGT; primitive gut tube, PFG; posterior foregut, PP; pancreatic progenitor. (B) Schematic procedure for the generation of pancreatic organoids. Representative images of 585A1 cells are shown. Scale bars, 200 μ m (C) Representative S4D5 organoids. Scale bars, 200 μ m. (D) Immunohistochemical characterization of S4D5 organoids. Scale bars, 100 μ m. (E) Representative flow cytometry plots and (F) Percentage of PDX1 + /NKX6.1 + cells in S4D5 organoids. Data are presented as mean $\pm$ SD from five (585A1) and three (604B1) independent experiments. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

sequencing was confirmed by Applied Biosystems 3500xL (Thermo Fisher Scientific).

2.6. Flow cytometry

Organoids were released into single-cell suspension using Accumax (ICT) at 37 °C for 30 min. Cells were then washed twice in FACS buffer (PBS(-) containing 10 μ M Y-27632 and 0.2 % FBS). Cells were first incubated with LIVE/DEAD Fixable Blue dead cell stain kit (Invitrogen) for 15 min at 4 °C to allow for live/dead cell discrimination during analysis. For GP2 staining, conjugated primary antibodies were added to the cells and incubated. Stained cells were washed twice in FACS buffer and analyzed with a BD FACS Aria II. The reaction conditions of conjugated antibodies are listed in Supplemental Table 2. For intracellular protein staining, cells were fixed in 350 μ l of Cytofix/Cytoperm Buffer (BD) for 30 min at 4 °C, followed by two washes in Perm/Wash Buffer Solution (BD). Cells were re-suspended in 2.5% normal horse serum for 10 min at room temperature. Cells were then incubated for 1 h at room temperature with primary antibodies or isotype control IgG at the same concentration, followed by two washes in Perm/Wash buffer. Finally, cells were incubated with the appropriate secondary antibodies for 30 min at room temperature, followed by two washes prior to analysis on the BD FACS Aria II. The concentrations of primary and secondary antibodies are listed in Supplemental Table 2. Cells were analyzed on BD FACS Aria II using BD FACS Diva software after the acquisition of at least 10,000 events.

2.7. EdU incorporation

Cells were incubated in culture medium containing 10 μ M EdU for 12 hours, and processed using the Click-iT EdU Alexa Fluor 647 Imaging Kit (Thermo Fisher Scientific). Labeled cells were analyzed on a BD FACS Aria II using the BD FACS Diva software, with at least 10,000 events being acquired.

2.8. Glucose-stimulated C-peptide secretion tests

Differentiated organoids were preincubated for 1 h at 37 °C in Krebs-Ringer bicarbonate HEPES buffer (KRBH; 116 μ M NaCl, 4.7 μ M KCl, 2.5 μ M CaCl₂, 1.2 μ M KH₂PO₄, 1.2 μ M MgSO₄, 24 μ M HEPES, 25 μ M NaHCO₃, and 0.1% BSA) containing 2 mM glucose. Then, the organoids were incubated at 37 °C in KRBH containing 2 mM glucose for 1 h, followed by 22 mM glucose for 1 h. Finally, the organoids were incubated for 1 h at 37 °C in KRBH containing 2 mM glucose and 30 μ M potassium chloride. C-peptide was measured with a human C-peptide ELISA kit (Mercodia AB, Uppsala, Sweden).

2.9. Transplantation experiments

All animal experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University. Female, 15 to 28 weeks old, euglycemic NOD.Cg-Rag1^{tm1Mom}Il2rg^{tm1Wjl}/SzJ mice (NRG, Charles River Laboratories Japan) were maintained on a 12 h light/dark cycle with *ad libitum* access to a standard irradiated diet. Mice were anesthetized with inhalable isoflurane. Stage 6 organoids, around 3.0×10^6 cells each, were implanted under the left kidney subcapsule. After 2 months, the mice were euthanized, and serial sections of the grafts were examined by immunostaining. Graft function was assessed by measuring human C-peptide levels in mouse plasma after glucose administration. The mice fasted for 6 hours, and 30% glucose was administered by intraperitoneal injection at a dose of 3.0 g/kg body weight. Blood samples from the jugular vein were collected into heparinized capillaries prior to and 30 min after glucose administration. The plasma human C-peptide level was analyzed by an ELISA kit (Mercodia). All metabolic analyses were performed in conscious, restrained animals.

2.10. Statistical analysis

All results are presented as mean $\pm$ SD. Data were analyzed using Student's *t*-test for two groups or one-way ANOVA followed by Tukey's test when multiple groups were compared. *P* < 0.05 was considered statistically significant. Statistical analyses were performed using JMP Pro 14 Software (SAS Institute)

3. Results

3.1. Differentiation of hiPSCs into PDX1 + /NKX6.1 + pancreatic organoids

By modifying the previously reported protocol (Kunisada et al., 2012; Rezania et al., 2014; Pagliuca et al., 2014; Takeuchi et al., 2014; McCracken et al., 2017), we used human iPS cell lines 585A1 and 604B1 to establish a six-stage stepwise differentiation protocol (Fig. S1A and Fig. 1A (extracted from Stage 1 to Stage 4)). At the end of Stage 1, around 70% cells were SOX17 + / FOXA2 + definitive endoderm cells ($71.1 \pm 0.9\%$ and $72.4 \pm 1.9\%$ in 585A1 and 604B1, respectively) (Fig. S1B and C). At the end of stage 2 when approximately 80% of cells were HNF1B + /FOXA2 + primitive gut tube cells ($75.6 \pm 3.2\%$ and $81.8 \pm 0.9\%$ in 585A1 and 604B1, respectively) (Fig. S1D and E), we dissociated cells into single-cell suspensions and reseeded on a new dish. After 24 hours (at S3D1), the reseeded cells formed small clumps (Fig. 1B, inset). On day 1 stage 4 (S4D1), we scraped off the cell clumps, dispersed them by gentle pipetting, and transferred the cells into low-adherent plates. Further differentiation proceeded in a three-dimensional manner. Cell clumps formed three-dimensional clusters on S4D2. At this stage, clusters consisted of mainly PDX1 + cells ($53.3 \pm 8.0\%$ and 87.1% in 585A1 and 604B1, respectively), but almost no NKX6.1 + cells ($1.2 \pm 0.3\%$ and 0.9% in 585A1 and 604B1, respectively) (Fig. S1F). At S4D5, cell clusters were almost uniform in appearance and size (> 200 μ m diameter). Immunohistochemical and FACS analyses revealed that nearly 90% of cells expressed PDX1 ($89.0 \pm 0.65\%$ and $97.2 \pm 1.3\%$ in 585A1 and 604B1, respectively), with 50-60% co-expressing NKX6.1 ($48.9 \pm 3.1\%$ and $60.2 \pm 6.3\%$ in 585A1 and 604B1, respectively) (Fig. 1D,E,F, and S1G).

3.2. Transient FOXO1 inhibition results in enhanced production of NGN3 + pancreatic endocrine precursors

Since FoxO1 depletion causes huge islet formation in *Pdx1*-Cre-mediated FoxO1 KO mice (Talchai and Accili, 2015), we speculated that FoxO1 inhibition in pancreatic progenitors would favor enhanced NGN3 + cell production. We treated our pancreatic organoid with AS1842856 on S4D5, a chemical compound that moves the FoxO1 protein from the nucleus to cytoplasm, thereby inhibiting its transcriptional activity (Nagashima et al., 2010). 24 hours later, we added DAPT, a γ -secretase inhibitor, to stimulate *Ngn3* transcription, and cultured for three days (Fig. 2A). However, this AS1842856 treatment for 4 days resulted in decreased NGN3 mRNA expression (Fig. 2B). Moreover, the expression of other pancreatic genes, including *PDX1*, *NKX6.1*, and *SOX9*, significantly decreased (Fig. 2C). We noticed that the size of AS1842856-treated organoids was larger at S5D3 (Fig. 2D), suggesting FOXO1 inhibition causes cell proliferation.

Next, we treated S4D5 organoids with AS1842856 only for 24 hours (Fig. 2E). Interestingly, we observed downregulation of *PDX1*, *NKX6.1*, and *SOX9* during AS1842856 treatment at S4D6, which recovered 3 days after AS1842856 removal (Fig. 2F and S2A). Transiently decreased pancreatic gene expression during FoxO1 inhibition was observed in explant culture using E11.5 mouse embryonic pancreatic tissue (Fig. S2B), indicating conserved machinery between mouse and human pancreatogenesis. Notably, transient AS1842856 treatment resulted in increased NGN3 mRNA expression at S5D3 (3.9 ± 1.2 -fold and 10.7 ± 4.2 -fold in 585A1 and 604B1, respectively) (Fig. 2G). This

increase in *NGN3* mRNA expression is well explained by the increased *NGN3*+ cells at S5D3 as evidenced by our immunohistochemical and FACS analyses ($3.1 \pm 0.25\%$ vs. $8.8 \pm 0.74\%$; 2.8 fold and $2.9 \pm 0.37\%$ vs. $16.3 \pm 2.6\%$; 5.8 fold in 585A1 and 604B1, respectively) (Fig. 2H, I, and J). We observed no difference in the size or the ratio of dead cells between AS1842856-treated and control organoids at S5D3 (Fig. S3). To test if transient AS1842856 treatment stimulated *NGN3* transcription, we established an *NGN3*-EGFP reporter from the 604B1 line (Fig. S4, and see Materials and Methods). We FACS sorted the EGFP+ cells (*NGN3*+ cells) at S5D3 (Fig. 2K) and analyzed the *NGN3* expression. qPCR analyses of FACS sorted EGFP+ cells showed that the *NGN3* mRNA expression was the same in AS1842856-treated and DMSO-treated EGFP+ cells (Fig. 2L), indicating that pretreatment with AS1842856 did not stimulate *NGN3* transcription.

Ngn3 is expressed not only in pancreatic endocrine progenitors but also in enteroendocrine progenitor cells (Jenny et al., 2002). As S4D5 organoids contained approximately 50% *PDX1*+/*NKX6.1*+ pancreatic epithelium (Fig. 1F), we investigated the effects of transient AS1842856 treatment on pancreatic cells versus non-pancreatic cells. We separated glycoprotein 2 (GP2)+ pancreatic endoderm (Ameri et al., 2017; Cogger KF et al., 2017; Ramond et al., 2017; Ramond et al., 2018) and GP2- non-pancreatic cells from S4D5 organoids induced from 585A1 cell lines (Fig. 2M). The percentage of GP2+ cells ($42.4 \pm 6.4\%$, Fig. 2N) was comparable to *PDX1*+/*NKX6.1*+ cells ($44.0 \pm 1.9\%$, Fig. 2O). Relative *PDX1* and *NKX6.1* expression in GP2+ and GP2- populations were consistent with previous reports (Ameri et al., 2017) (Fig. 2P). After sorting, cells were transferred to low-adherent, V-shaped bottom dishes and treated with DMSO or AS1842856 for 24 h. Both GP2+ and GP2- cells formed cell clusters on the next day (Fig. 2Q). We found that AS1842856 stimulated the proliferation of both GP2+ and GP2- cells (Fig. 2R and S). However, upregulated *NGN3* expression (Fig. 2T) and the increase in the number of EGFP+ cells (Fig. S5) were observed in GP2+ spheres but not in GP2-negative, non-pancreatic spheres at S5D3.

3.3. Transient FOXO1 inhibition stimulates cell proliferation before the onset of *NGN3* expression

To evaluate the time course of *NGN3*+ cell production, we used *NGN3*-EGFP reporter cells and performed FACS analyses every 24 hours from S4D6 to S5D3. As shown in Fig. 3A and B, the EGFP+ cell ratio significantly increased by transient FOXO1 inhibition at S5D2 and S5D3 ($0.1 \pm 0.04\%$, $0.3 \pm 0.08\%$, $10.6 \pm 1.6\%$, and $25.3 \pm 4.2\%$ at S4D6, S5D1, S5D2 and S5D3, respectively). Immunostaining for *NGN3* revealed that $76.3 \pm 3.2\%$ of EGFP+ cells were *NGN3*-positive at S5D2, which decreased to $56.2 \pm 6.2\%$ at S5D3 (Fig. 3C and D), consistent with the longer EGFP half-life, compared to *NGN3* (Miyatsuka et al., 2011; Liu et al., 2014).

Because EGFP+ cells significantly increased between S5D1 and S5D2, we labeled cells with EdU at the latter half of S5D1 for 12 hours, and then analyzed the cells at S5D2 (Fig. 3E). We found that only $1.5 \pm 0.9\%$ EGFP+ cells were EdU+ in AS1842856-treated organoids (Fig. 3F and G), indicating a low proliferative activity of *NGN3*+ cells. This finding is compatible with the previous reports on mouse pancreatic development that showed the extremely low proliferative nature of *Ngn3*+ cells (Desgraz and Herrera, 2009; Miyatsuka et al., 2011). Thus, it is more likely that activated proliferation by transient FOXO1 inhibition precedes the onset of *NGN3* expression. To test this idea, we next labeled cells with EdU at the latter half of S4D5 for 12 hours, and then analyzed the cells at S5D2 (Fig. 3H). We found the percentage of labeled cells among EGFP+ cells significantly increased by AS treatment; $61.1 \pm 11.1\%$ and $35.4 \pm 2.4\%$ in DMSO-treated and AS-treated samples, respectively (Fig. 3I and J), indicating the significant contribution of proliferating cells as the origin of *NGN3*+ cells.

Taken together, these findings indicate that transient FOXO1 inhibition stimulates the proliferation of GP2+ cells that gives rise to

NGN3+ endocrine precursors.

3.4. FoxO1 inhibition does not affect Notch activity but activates Wnt signaling

It is reported that *Hes1* suppresses *MyoD* (Kitamura et al., 2007) and *Ngn3* (Ahnfelt-Ronne et al., 2012) expression, thereby inhibiting myogenic and pancreatic endocrine differentiation, respectively. In myogenic differentiation, *FoxO1* and *Notch-IC* cooperatively upregulate *Hes1* transcription (Kitamura et al., 2007). However, AS1842856 treatment for 24 hours did not change *HES1* expression at S4D6 (Fig. 4A), while subsequent DAPT treatment for 3 days significantly suppressed *HES1* expression at S5D3.

We found that FOXO1 inhibition increased expression of Wnt target genes, including *AXIN2* (Behrens et al., 1998; Huch et al., 2013), *TCF-1* (Tiemessen et al., 2012; Toyoda et al., 2015; De Jaime-Soguero et al., 2017) and *LEF-1* (Hovanes et al., 2001; Filali M et al., 2002). Increased expressions of *AXIN2* and *LEF-1* were suppressed by the Wnt inhibitors XAV939 with PKF118-310, whereas Wnt inhibitors did not suppress the increased *TCF-1* expression by an unknown cause (Fig. 4B and C). Notably, the increased *NGN3* expression was significantly suppressed by Wnt inhibitors, indicating that the AS1842856 effect was mediated by Wnt activation (Fig. 4D and Fig. S6). This corroborates our finding that transiently activating Wnt using CHIR99021 results in the comparable upregulation of Wnt target genes and *NGN3* at S4D6 and S5D3, respectively (Fig. 4B, C, and D). These findings are compatible with previous reports that FoxO proteins and TCF/LEF transcription factors are competitive nuclear β -catenin binding proteins (Almeida et al., 2007; Essers et al., 2005; Hoogeboom et al., 2008). Indeed, the expression of FOXO1 target genes *PDK4* (Shin et al., 2012; Gopal et al., 2017) and *HBP1* (Watanabe et al., 2015; Chan et al., 2017) significantly decreased by either AS1842856 or CHIR99021 at S4D6 (Fig. 4E). Decreased *PDK4* and *HBP1* expression levels by AS1842856 treatment were not rescued by XAV939 with PKF118-310, because AS1842856 locates FOXO1 to the cytoplasm (Nagashima et al., 2010) where it cannot bind to the *PDK4* promoter region. Furthermore, we provide evidence of the opposite effects of FOXO1 and Wnt in murine pancreas organogenesis. *Axin2* expression was elevated in AS1842856-treated murine embryonic pancreatic tissue at E11.5, which was inhibited by XAV939 with PKF118-310. In addition, activation of Wnt signaling by CHIR99021 suppressed *Hbp1* (Fig. S7).

To confirm that the cell proliferative effect by AS1842856 was mediated through Wnt activation, we labeled S4D5 cells with EdU for 12 hours (Fig. 4F). We observed that the number of EdU-labeled cells increased by CHIR99021 as effectively as AS1842856 ($37.5 \pm 1.7\%$ in control, $51.3 \pm 2.5\%$ and $59.4 \pm 5.9\%$ in AS1842856-treated and CHIR99021-treated organoid, respectively). Wnt inhibition via XAV939 with PKF118-310 inhibited the AS1842856 effect on cell proliferation (reduced to $24.1 \pm 7.7\%$ in EdU labeling; Fig. 4G and H).

3.5. Characterization of the pancreatic organoid induced by transient FoxO1 inhibition protocol

We analyzed the characteristics of the final organoid product of our transient FOXO1 inhibition protocol (Fig. 5A). AS1842856-treated organoids mainly consisted of Synaptophysin+ endocrine cells and CK19+ duct cells (Fig. 5B), while Trypsin+ exocrine cells were rarely detected (data not shown). As expected, transient FOXO1 inhibition significantly increased endocrine cells (Fig. 5C). The ratios of Insulin (INS)+ or Glucagon (GCG)+ cells were $4.3 \pm 1.8\%$ vs. $12.5 \pm 2.5\%$ and $8.8 \pm 2.7\%$ vs. $25.3 \pm 7.3\%$ in 585A1 and 604B1 respectively, and all endocrine cells subpopulations we analyzed, including INS+/GCG-, INS+/GCG+, and INS-/GCG+ cells, significantly increased by transient FOXO1 inhibition (Fig. 5D and E). When transplanted into the renal subcapsule space of NRG mice, endocrine cells from AS1842856-treated organoids became monohormonal, mainly GCG+

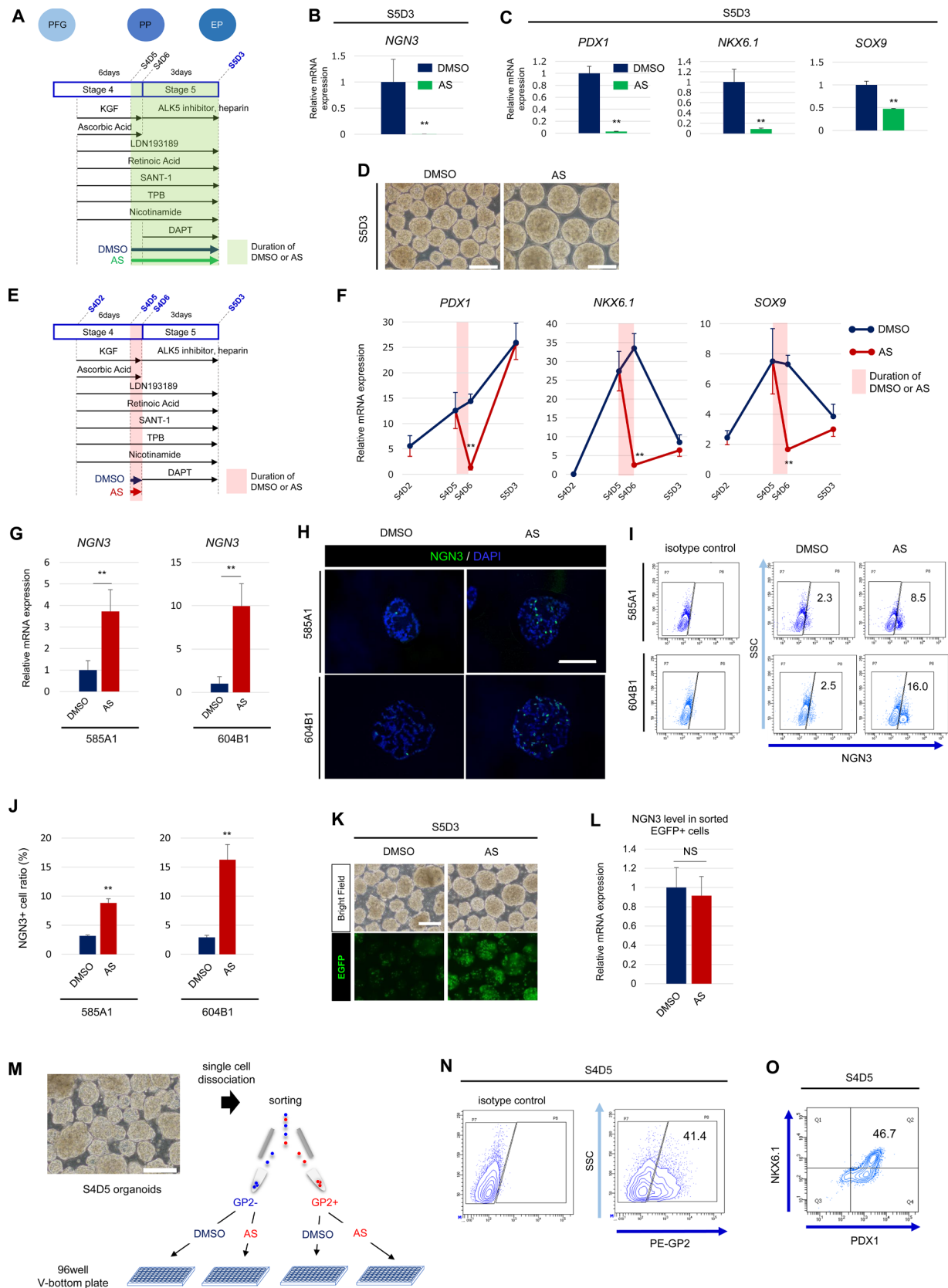


Fig. 2. Transient FoxO1 inhibition accelerates the induction of NGN3+ cells. (A) Overview of the protocol of Stage 4 and 5 for prolonged FoxO1 inhibition. EP, endocrine precursor; AS, AS1842856. (B and C) mRNA expression of *NGN3* and the indicated pancreatic multipotent genes at S5D3. (D) Representative S5D3 organoids. (E) Overview of the protocol of Stage 4 and 5 for transient FoxO1 inhibition. (F) mRNA levels of the indicated pancreatic multipotent genes from S4D2 to S5D3. The expression levels of the human adult pancreas were considered “1.” (G) mRNA expression of *NGN3* at S5D3. (H) Immunohistochemical analysis of S5D3 organoids. (I) Representative flow cytometry plots and (J) Percentage of NGN3+ cells in S5D3 organoids in each condition. Data represent five (585A1) and four (604B1) independent experiments. (K) S5D3 organoids using the NGN3-EGFP reporter in each condition. (L) NGN3 mRNA levels of FACS-sorted EGFP+ cells in each condition at S5D3. (M) Experimental procedures using the GP2 antibody. (N) Representative flow cytometry plots of GP2 and (O) PDX1+/NKX6.1+ cells at S4D5. (P) mRNA levels of the indicated genes of S4D5 GP2- and GP2+ sorted cells. The expression level of GP2- was considered “1.” (Q) Images of pooled spheres at S4D6 in each condition. (R) Ki67 staining of S4D6 spheres in each condition. (S) Percentage of Ki67+ cells in each condition. (T) mRNA levels of *NGN3* at S5D3 in each condition. Data in (B), (C), (F), and (N)–(T) were obtained using the 585A1 line. Data in K and L are from the NGN3-EGFP reporter cell line. The assays were performed in (B), (C), (F), (L), (P), (S) and (T) three, (J) five (585A1) and four (604B1), and (G) seven (585A1) and six (604B1) independent experiments and the expression levels are presented as mean $\pm$ SD. (B), (C), (F), (G), (J), (L) and (P) * p < 0.05, ** p < 0.01, Student's t -test. (S) and (T) * p < 0.05, ** p < 0.01, One-way ANOVA followed by Tukey's test. Scale bar, 100 μ m.

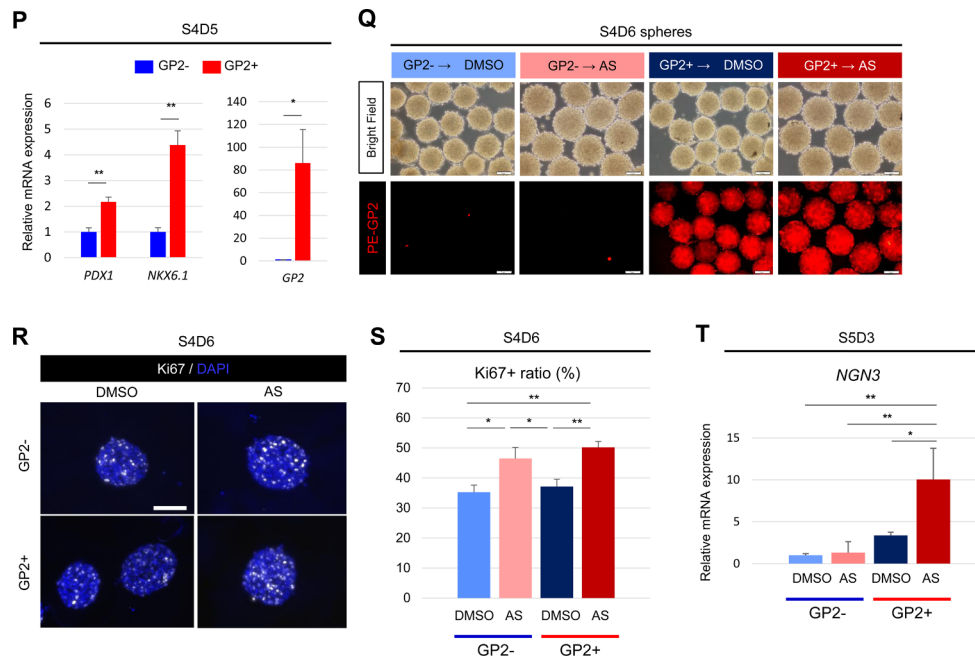


Fig. 2. (continued)

cells (Fig. 5F). These results are consistent with previous reports that INS+/GCG+ cells become GCG+ cells in human pancreatic development (Riedel et al., 2012). In addition, we observed CK19-expressing ductular structures and Trypsin+ primitive acinar cells in the graft tissue, indicating the presence of pancreatic epithelial cell progenitors within AS1842856-treated organoids (Fig. 5G). The organoids did not show apparent glucose responsiveness *in vitro* (Fig. S8A). We detected human C-peptide in the serum in 3 of 6 mice two months after transplantation, but they were not significantly elevated upon glucose challenge (Fig. S8B).

4. Discussion

In this study, we demonstrated that transient FOXO1 inhibition stimulates the proliferation of PDX1+/NKX6.1+ cells, resulting in elevated NGN3+ precursors and endocrine cells in pancreatic cells induced from human iPS cells. Produced insulin+ cells are not functionally matured; thus, the improvement of a differentiation protocol from NGN3+ cells to functional beta cells is required.

We provided evidence that FOXO1 inhibition has the same effect as Wnt activation in the differentiation of pancreatic endocrine cells from human iPS cells and in murine pancreatogenesis. Mutually opposing effects between FoxO protein activity and Wnt signaling were previously reported in osteogenesis, chondrocytogenesis, and cardiomyopathy pathogenesis in mice (Iyer et al., 2013; Matsuzaki et al., 2018; Okada et al., 2015). In murine pancreatic development, the hypoplastic

formation of exocrine tissue was reported in both Pdx1-Cre-mediated β -catenin knockout (Murtaugh, 2005) and enforced FoxO1 expression using Pdx1-promoter (Kitamura et al., 2009), indicating opposing effects of FoxO1 and Wnt signaling. However, we speculate that the underlying mechanism is much more complex than what has been uncovered so far. While FoxO1 inactivation using Pdx1-Cre strains exhibits a giant islet phenotype (Talchai and Accili, 2015), activation of Wnt signaling at an early stage (E10.5) decreases the number of endocrine cells, but Wnt activation at a late stage (E11.5) does not interfere with endocrine cell number (Heiser et al., 2006). In addition, Tet-off-mediated Wnt7b overexpression from E8.0 to E10.5 or E11.5 causes agenesis of insulin+ and glucagon+ cells, but endocrine differentiation was observed by Wnt7b overexpression from E8.0 to E9.5 (Afelik et al., 2015). Thus, interpretation of the mutant phenotype and the relationship between FoxO1 and Wnt signal requires careful attention, depending on the timing and/or duration of signaling activation/suppression and it may be connected to the gradual decrease in Wnt activity during pancreatogenesis (Afelik et al., 2015; Scavuzzo et al., 2018). Previous reports and our present study indicate that this notion is applicable to human pancreatic endocrine differentiation. Sharon N et al. (2019b) reported that Wnt inhibition in the pancreatic endoderm increases NGN3 expression in the differentiation protocol from human ES cells. At the same time, they show the lack of hormone expression in Ngn3-Cre;APC^{flxed} mouse, indicating that ectopic Wnt activation in Ngn3+ cells hinders proper endocrine differentiation. Al-Masri et al. (2010) reported that siRNA-based continuous

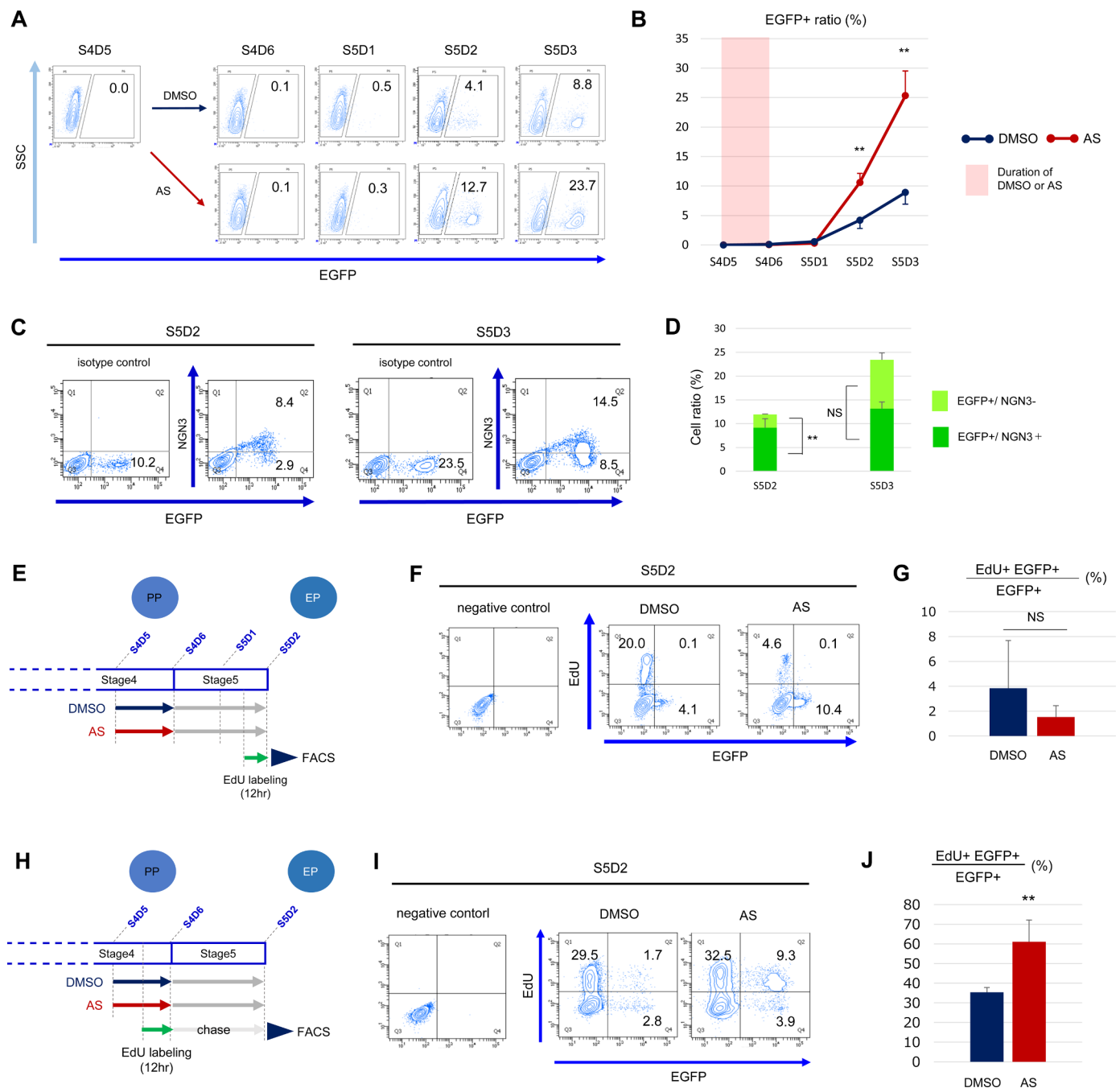


Fig. 3. Analysis using the NGN3 reporter cell line. (A) Sequential representative flow cytometry plots of EGFP+ cells and (B) time course of EGFP+ ratio. (C) Representative flow cytometry plots of EGFP+ and Alexa 647-labeled NGN3+ cells at S5D2 and S5D3, and (D) ratio of NGN3+ and NGN3- cells in EGFP+ cells. (E) The experimental schema for EdU labeling. (F) Representative flow cytometry plots of EGFP+ cells and Alexa 647-labeled EdU+ cells at S5D2. (G) EdU+ cell ratio in EGFP+ cells. (H) The experimental schema for EdU labeling and chasing. (I) Representative flow cytometry plots of EGFP+ cells and Alexa 647-labeled EdU+ cells at S5D2. (J) EdU+ cell ratio in EGFP+ cells. The assays were performed in (B), (D) and (G) three and (J) four independent experiments and presented as mean $\pm$ SD. ** p < 0.01, Student's t -test.

FOXO1 knockdown causes accelerated NKX6.1 and NGN3 expression in human embryonic islets obtained from 18-21 weeks old fetal tissue. Furthermore, FOXO1 inhibition, either by siRNA or AS1842856, during the induction of PDX1+ cells from human iPS cells increases NGN3 and insulin mRNA (Yu et al., 2018). Conversely, our results show that FOXO1 inhibition in PDX1+/NKX6.1+ cells has variable NGN3 expression, depending on the duration of AS1842856 treatment.

Our data suggest that FOXO1 inhibition results in a transient decrease in NKX6.1, followed by the proliferation of GP2+ cells that give rise to NGN3+ cells. We demonstrated that FoxO1 inhibition stimulates

PDX1+/NKX6.1+ cell proliferation. In this process, pancreatic epithelial markers, including PDX1, NKX6.1, and SOX9, are down-regulated in human iPS cell-based *in vitro* experiments and *ex vivo* mouse embryonic pancreas tissue culture, suggesting that the proliferation of PDX1+/NKX6.1+ cells is not simple self-duplication. Rather, it indicates a de-differentiation phenotype connected to FoxO1 and/or Wnt signaling. Moreover, a similar de-differentiation phenotype is observed in Wnt7b-overexpressing mice, as reflected by the reduced expressions of Nkx6.1, Ptf1a, and Ngn3 (Afelik et al., 2015). Interestingly, FoxO1 is involved in the δ -to- β cell transdifferentiation triggered

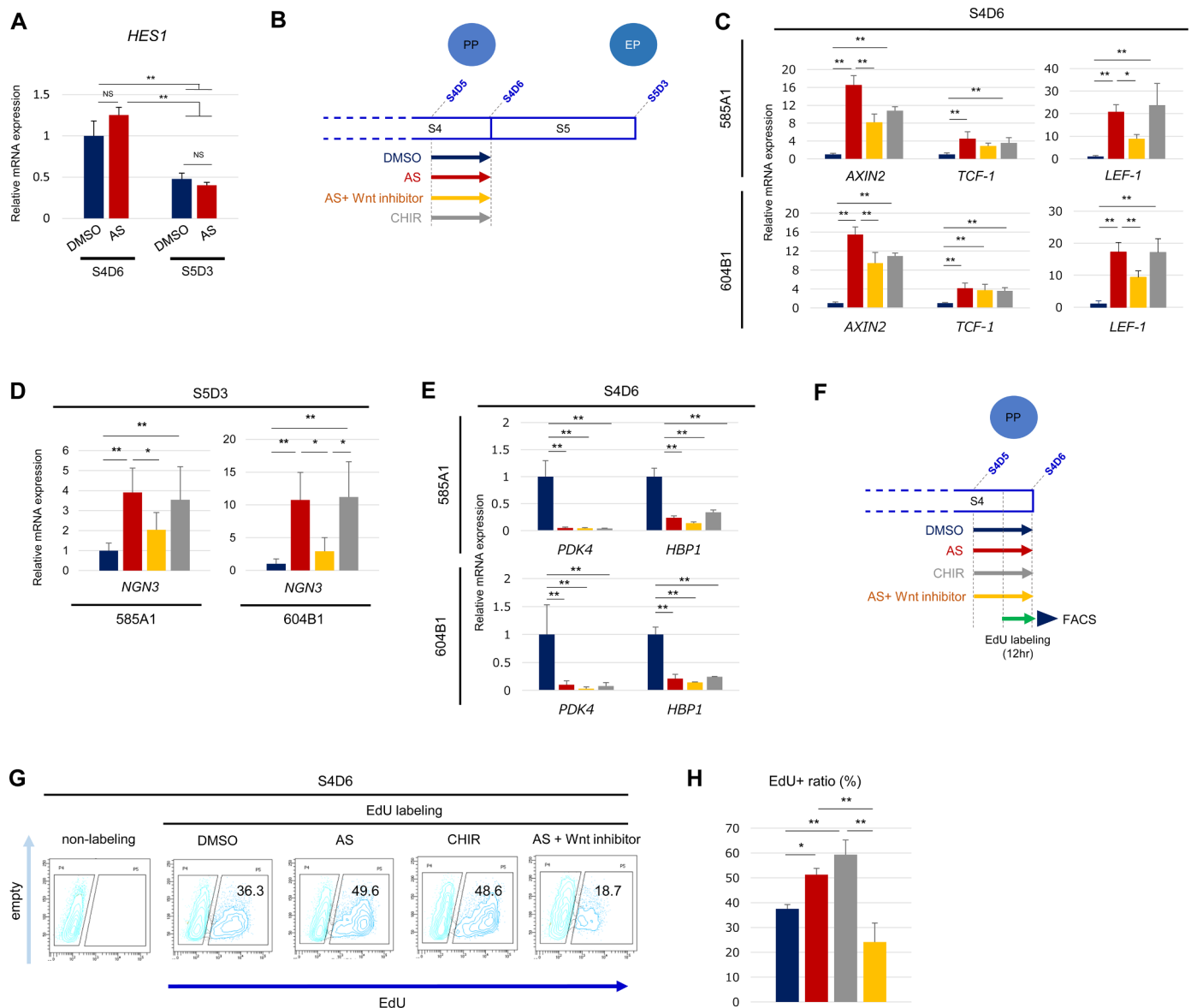


Fig. 4. Interaction between FoxO1 inhibition and the NOTCH/Wnt pathway. (A) mRNA levels of *HES1* at S4D6 and S5D3. (B) Experimental schema of each condition. CHIR; CHIR99021, Wnt inhibitors; XAV939 and PKF118-310. (C) mRNA levels of *AXIN2*, *TCF-1*, and *LEF-1* at S4D6. (D) mRNA levels of *NGN3* at S5D3. (E) mRNA levels of *PDK4* and *HBP1* at S4D6. (F) The experimental schema for EdU labeling. (G) Representative flow cytometry plots and (H) percentage of EdU+ cells in S4D6 organoids. The assays were performed in (A) and (C) *AXIN2*, *TCF-1*, and (E) three, (C) *LEF-1* and (H) four, (D) seven (585A1), and six (604B1) independent experiments, and the expression levels are presented as mean $\pm$ SD. * p < 0.05, ** p < 0.01, One-way ANOVA followed by Tukey's test.

by β cell ablation in murine islets. Chera et al. (2014) reported the detailed behavior of δ cells after β cell ablation. Somatostatin expression is reduced in these cells, while proliferation is stimulated, accompanied by antecedent FoxO1 reduction. They subsequently become Ngn3+ cells and further differentiate into β cells. This transdifferentiation is observed in 2-week-old juvenile δ cells, but not adult δ cells. However, transient FoxO1 inhibition by AS1842856 enables δ -to- β transdifferentiation in adult islets. These reports and our data indicate the ingenious mechanism that controls the proliferation and differentiation status of pancreatic cells during embryonic organogenesis and in adult islet plasticity, in which FoxO1 and/or Wnt signaling are tightly connected.

CRediT authorship contribution statement

Ben Sasaki: Conceptualization, Methodology, Validation, Formal analysis, Writing - original draft. **Shinji Uemoto:** Supervision. **Yoshiya**

Kawaguchi: Conceptualization, Writing - original draft, Supervision.

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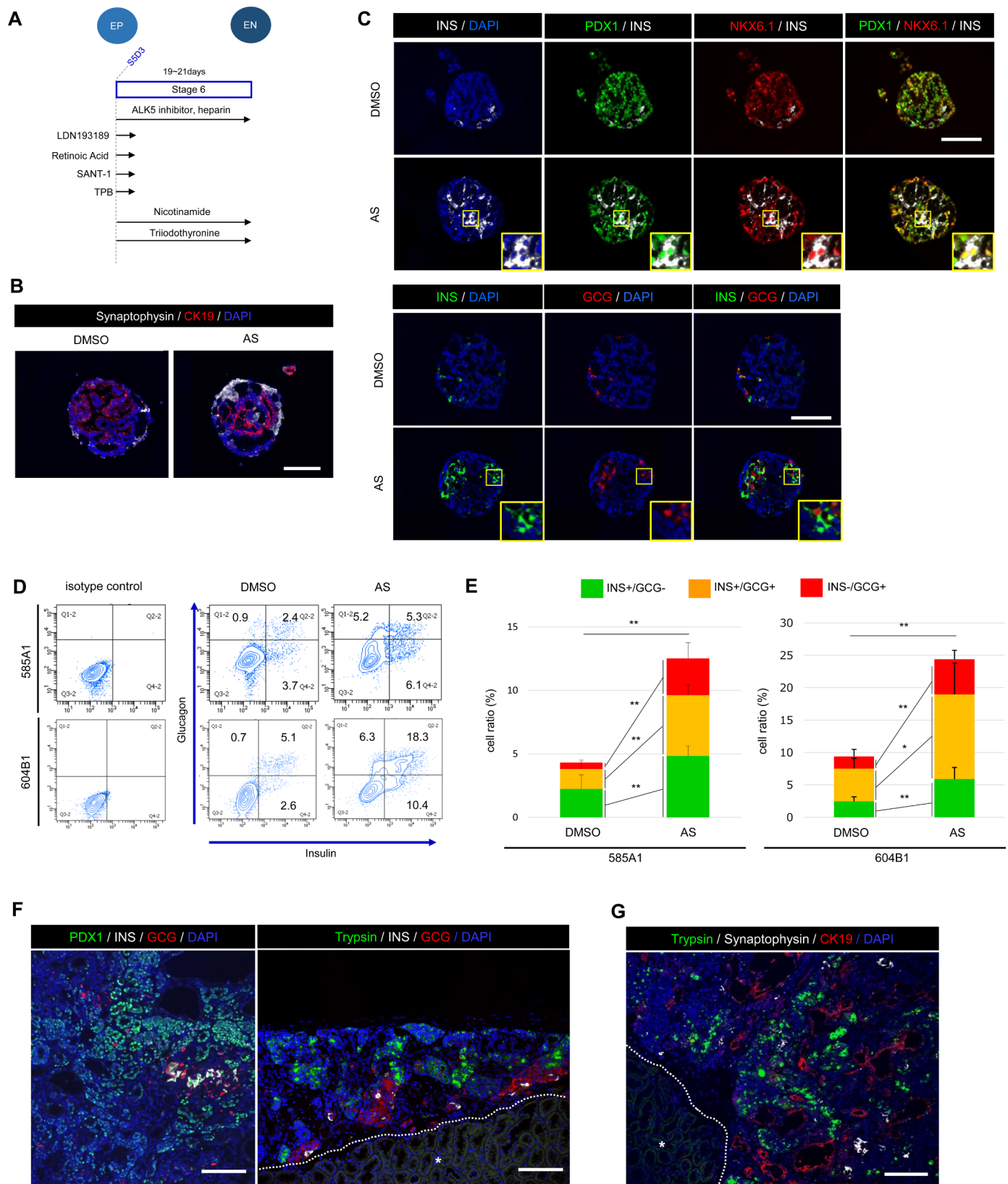


Fig. 5. Characterization of *in vitro* and *in vivo* S6 organoids. (A) Overview of the protocol of Stage 6. EN; endocrine cells. (B) Immunohistochemical image of AS1842856-treated organoids for synaptophysin and CK19. (C) Immunohistochemistry for PDX1/NKX6.1/INS and INS/GCG. (D) Representative flow cytometry plots of S6 organoids and (E) quantification. The assays were performed in five (585A1) or four (604B1) independent experiments, and their ratios are presented as mean $\pm$ SD. * p < 0.05, ** p < 0.01, Student's *t*-test. (F) and (G) Immunohistochemical analysis of AS-treated transplanted S6 organoids after 2 months. Asterisks denote the murine renal parenchyma. Scale bars, 100 μ m. Data in (B) and (C) were obtained using the 585A1 line.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.scr.2020.101754](https://doi.org/10.1016/j.scr.2020.101754).

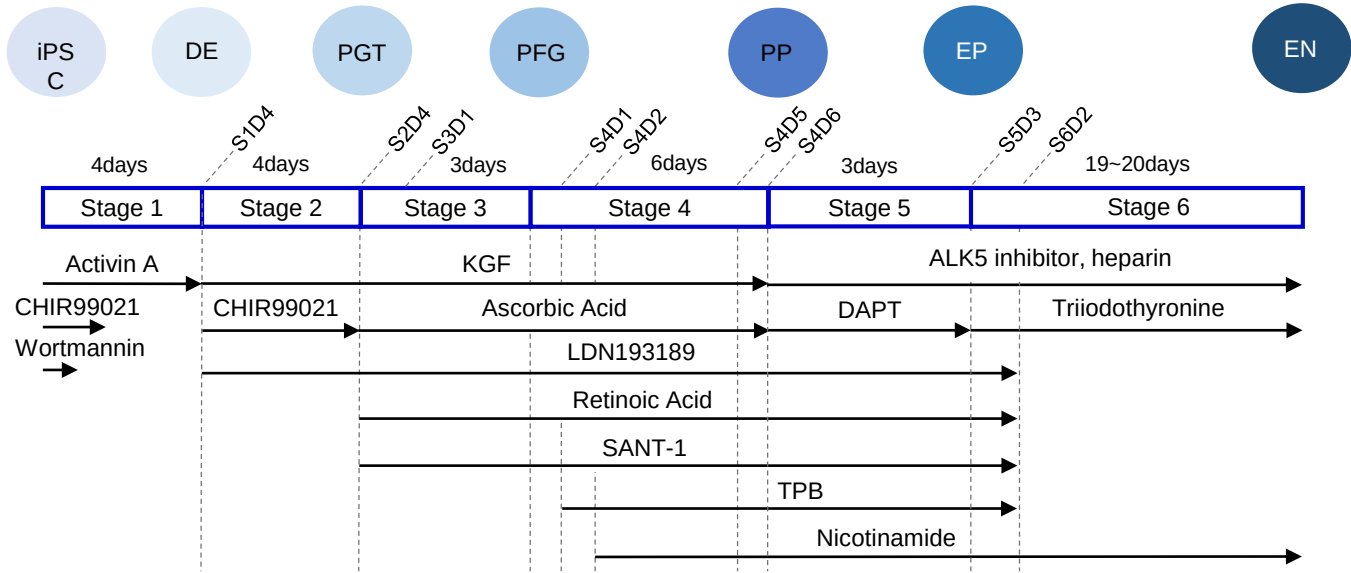
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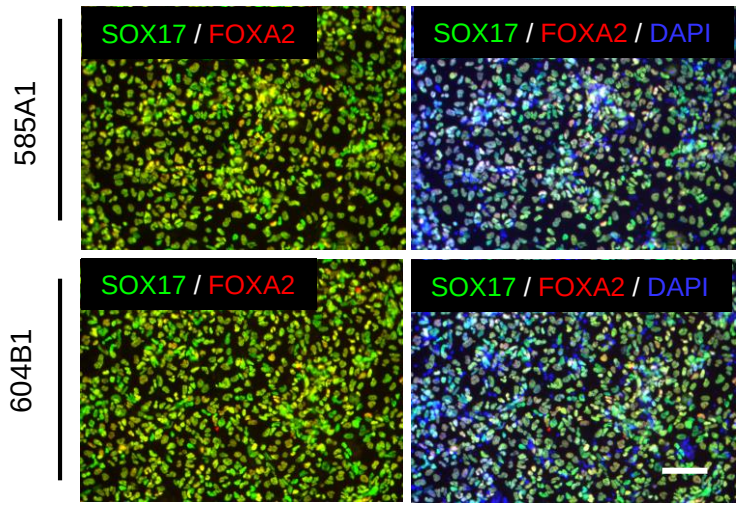
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Supplemental Figure. 1

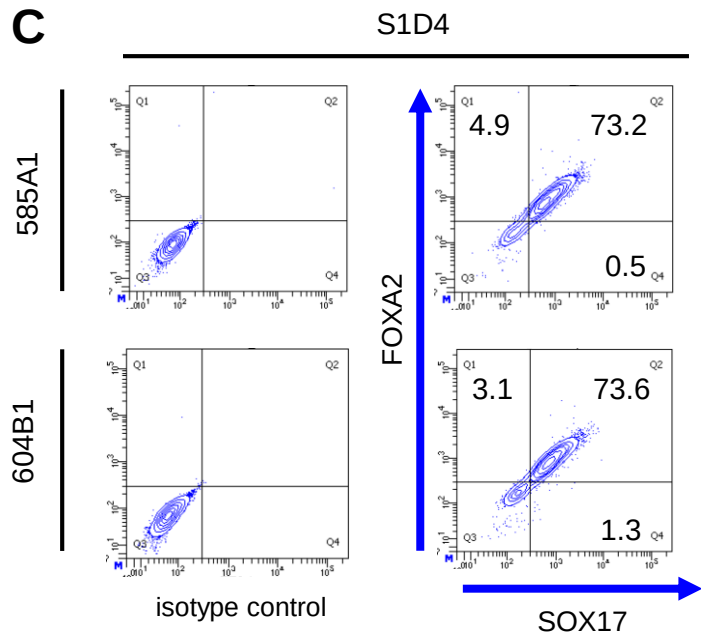
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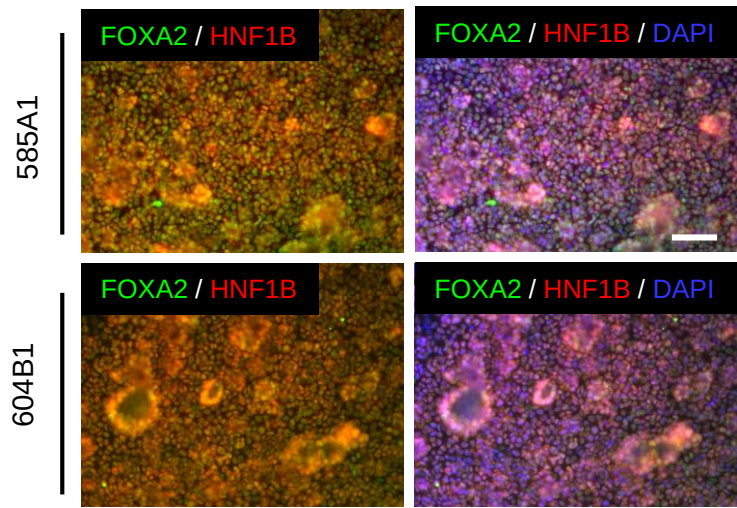
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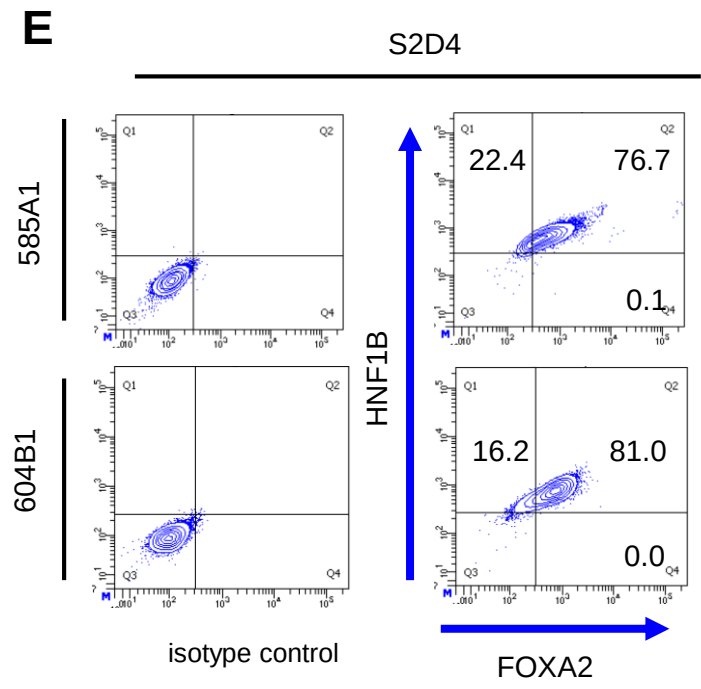
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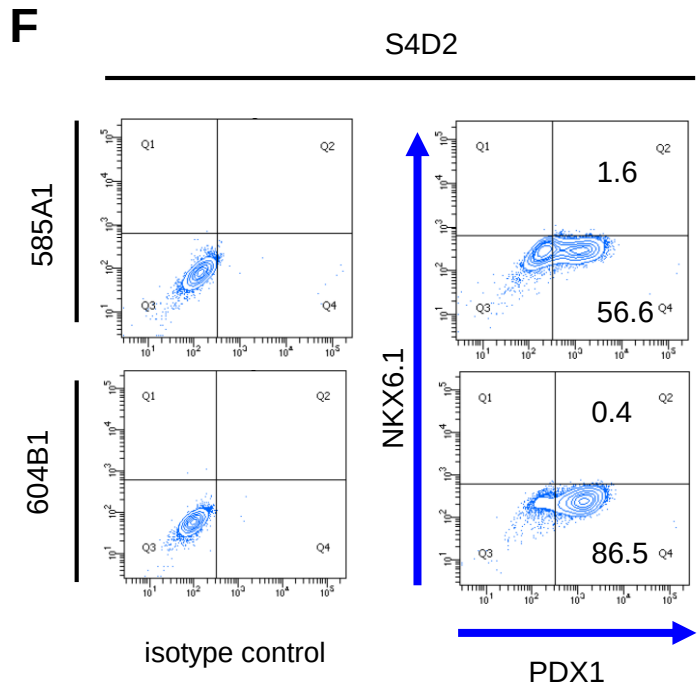
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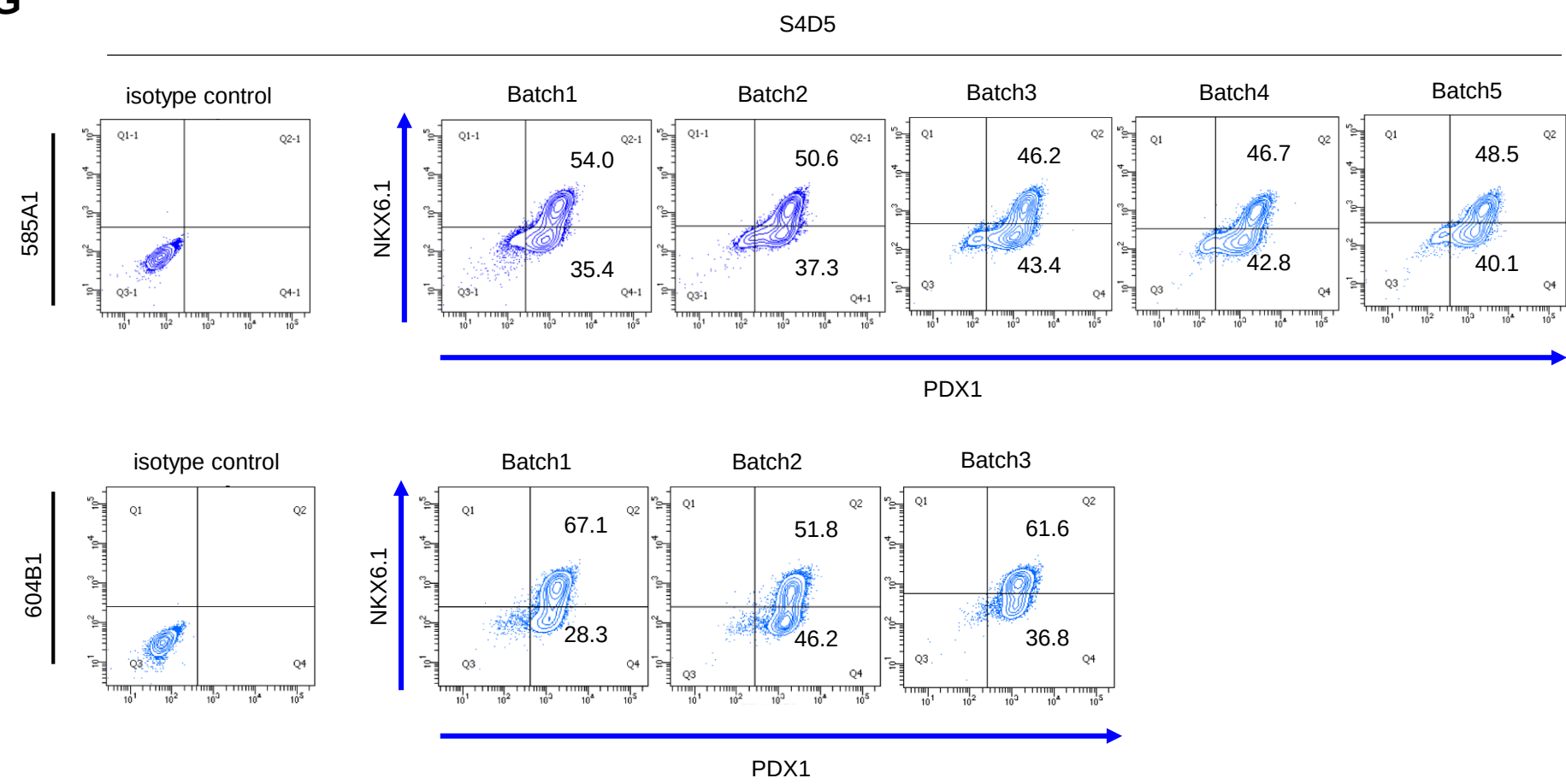
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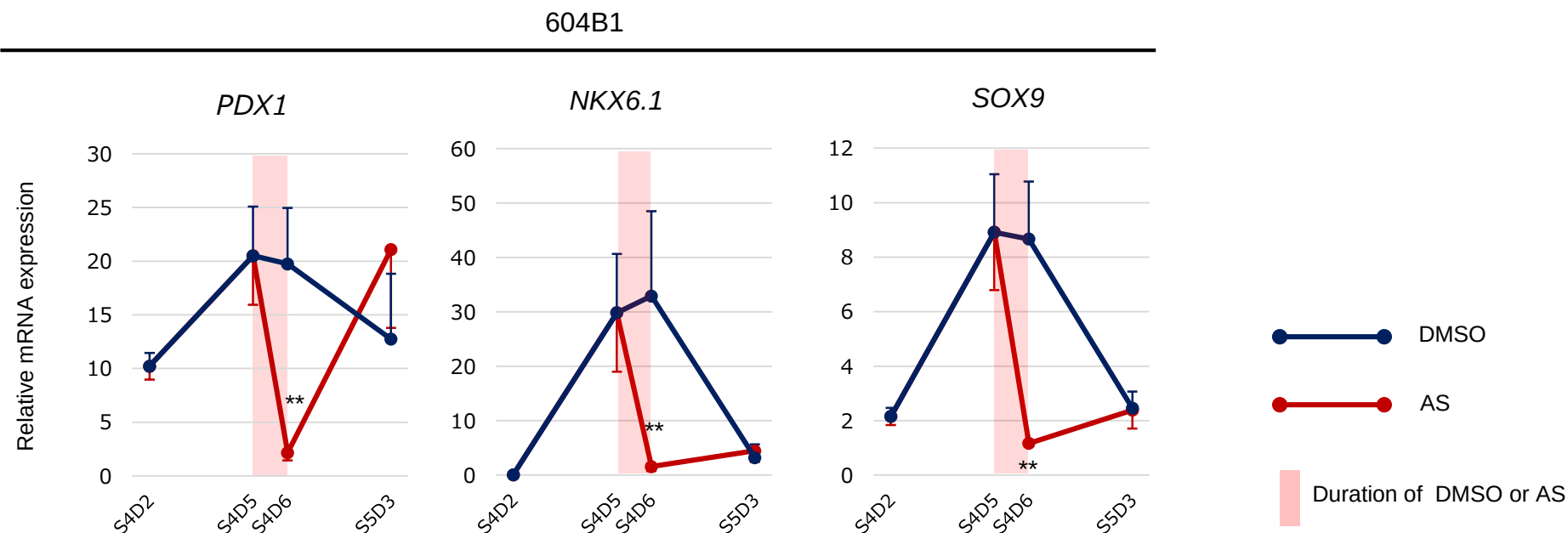


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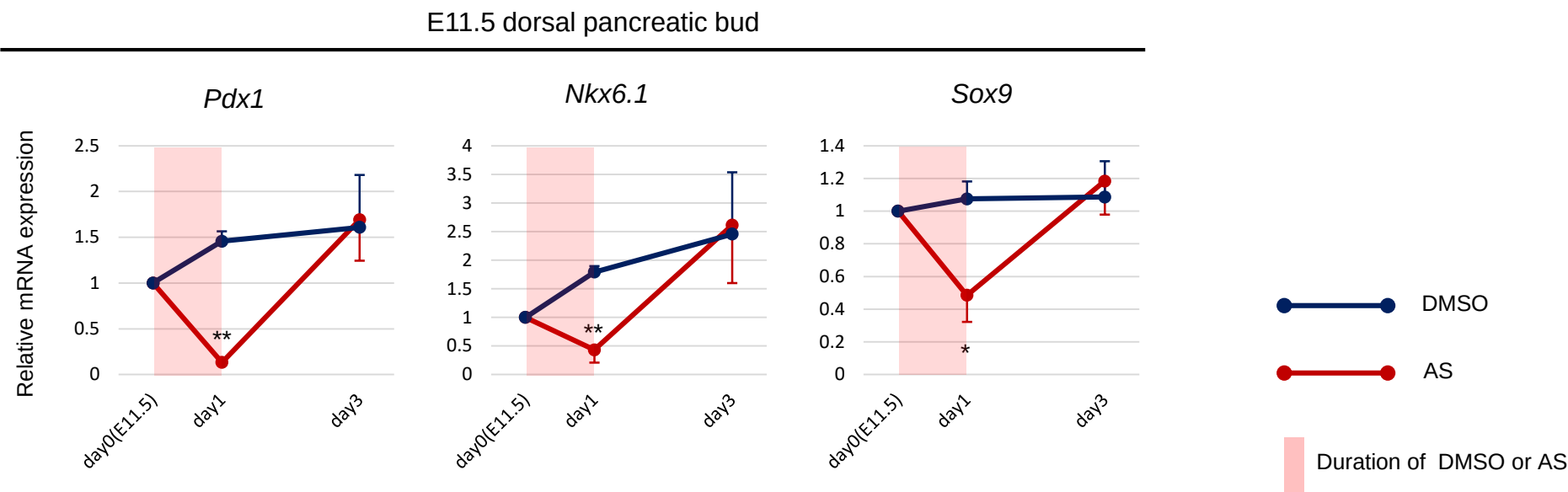


Supplemental Figure. 2

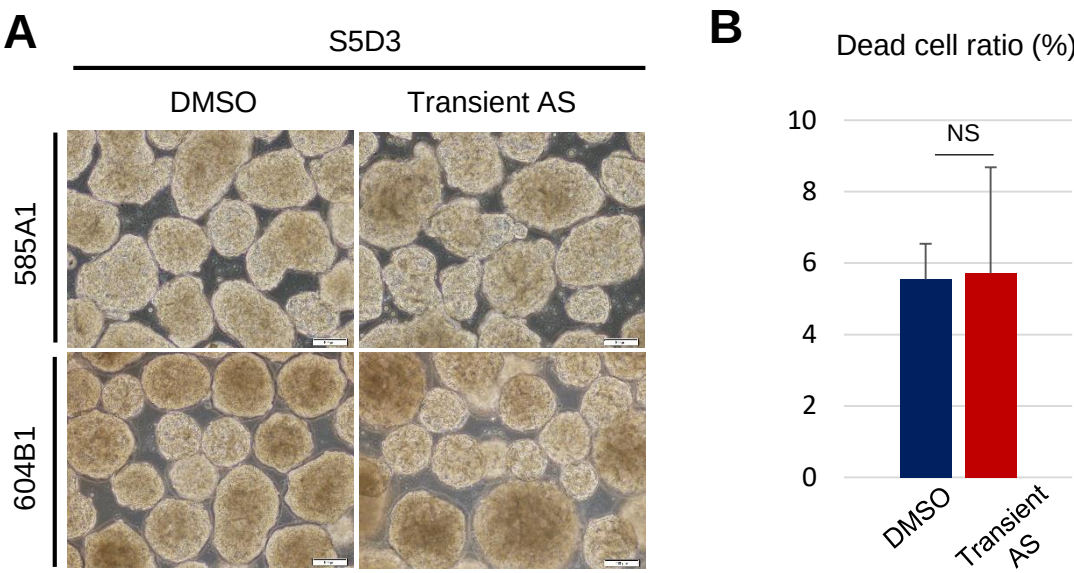
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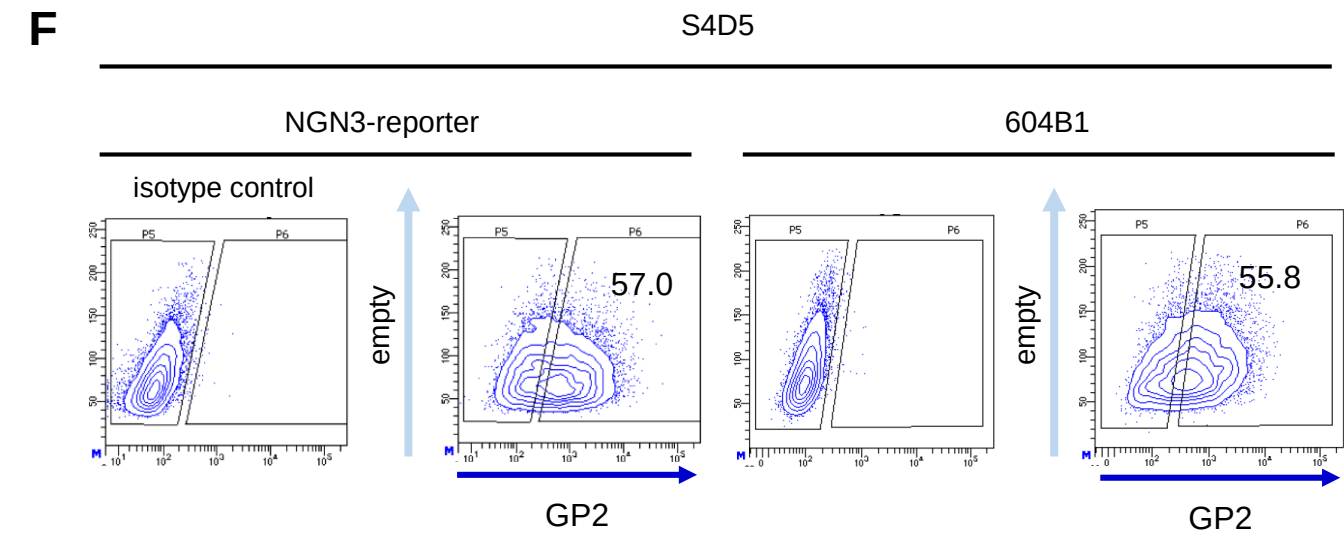
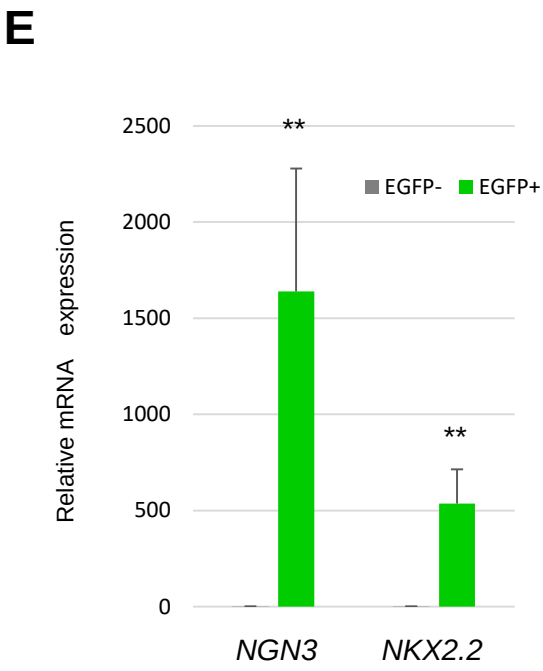
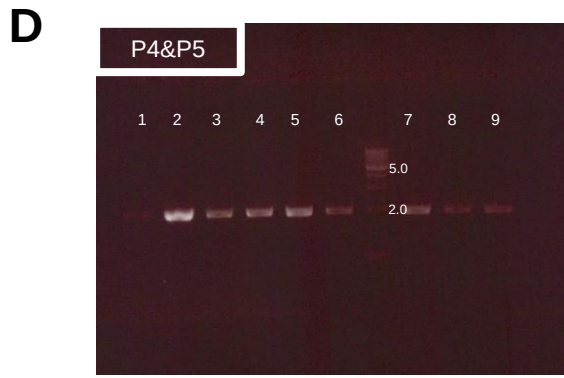
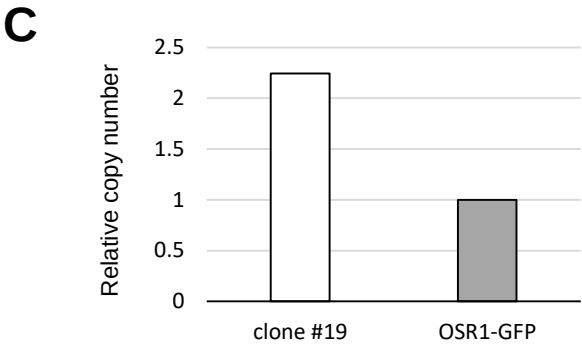
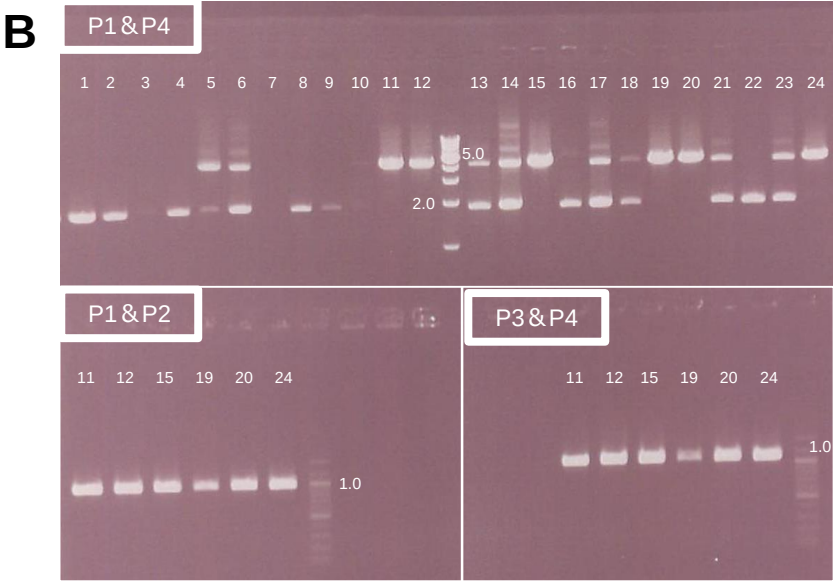
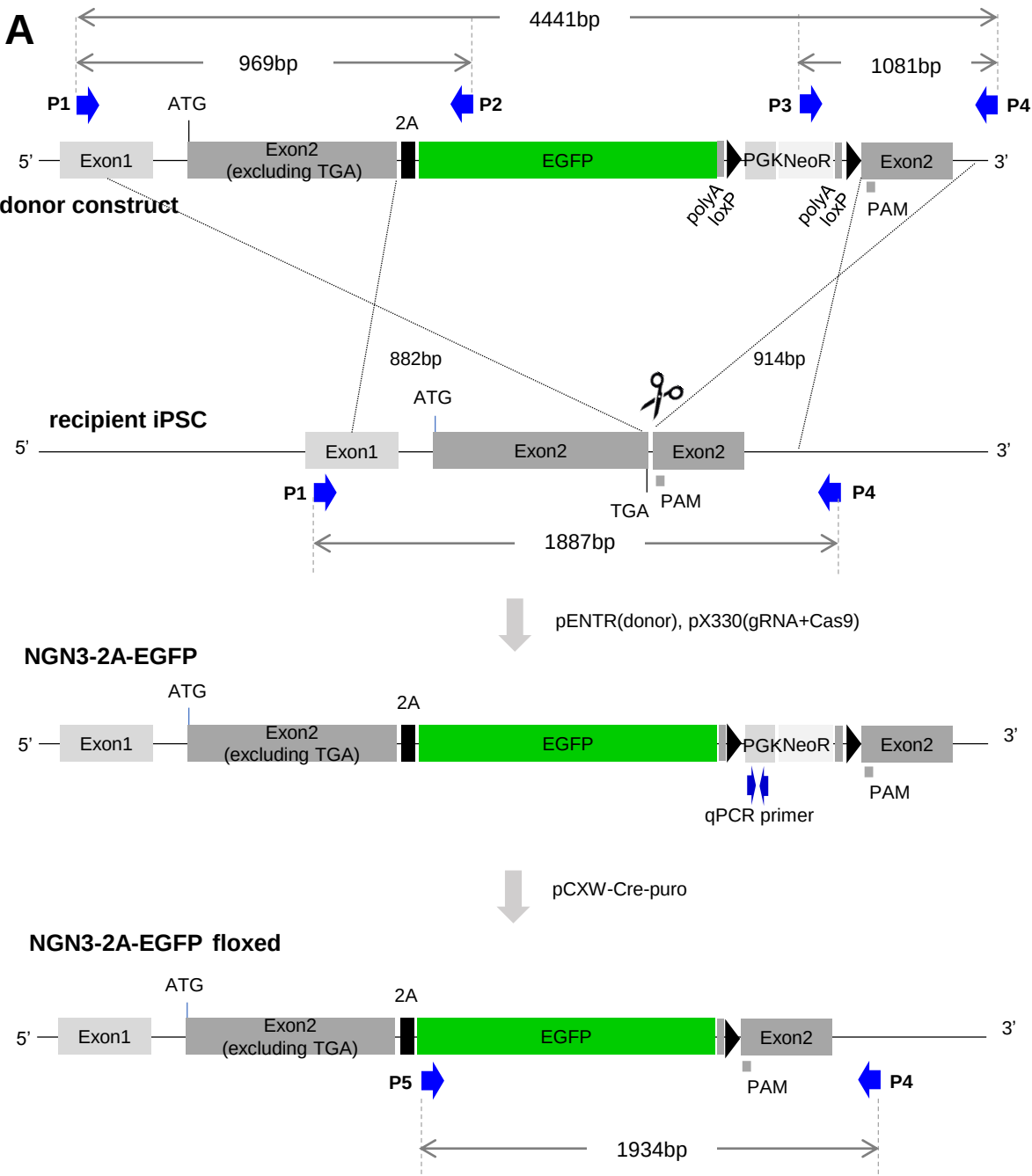
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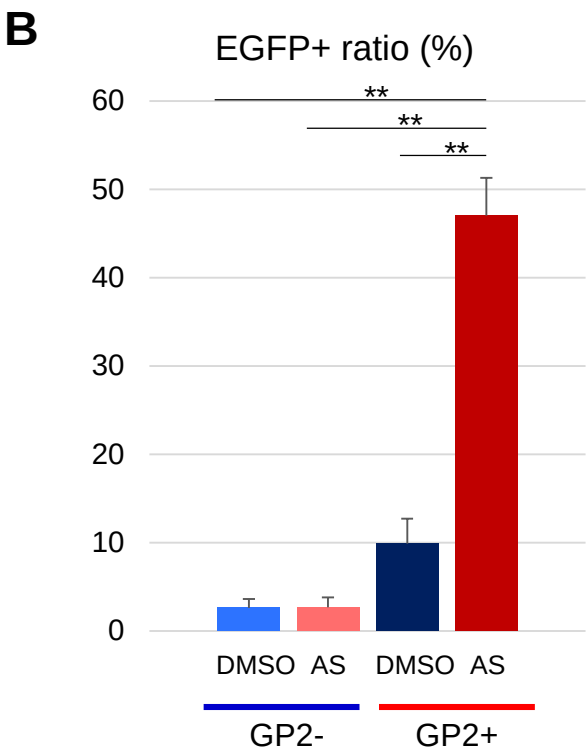
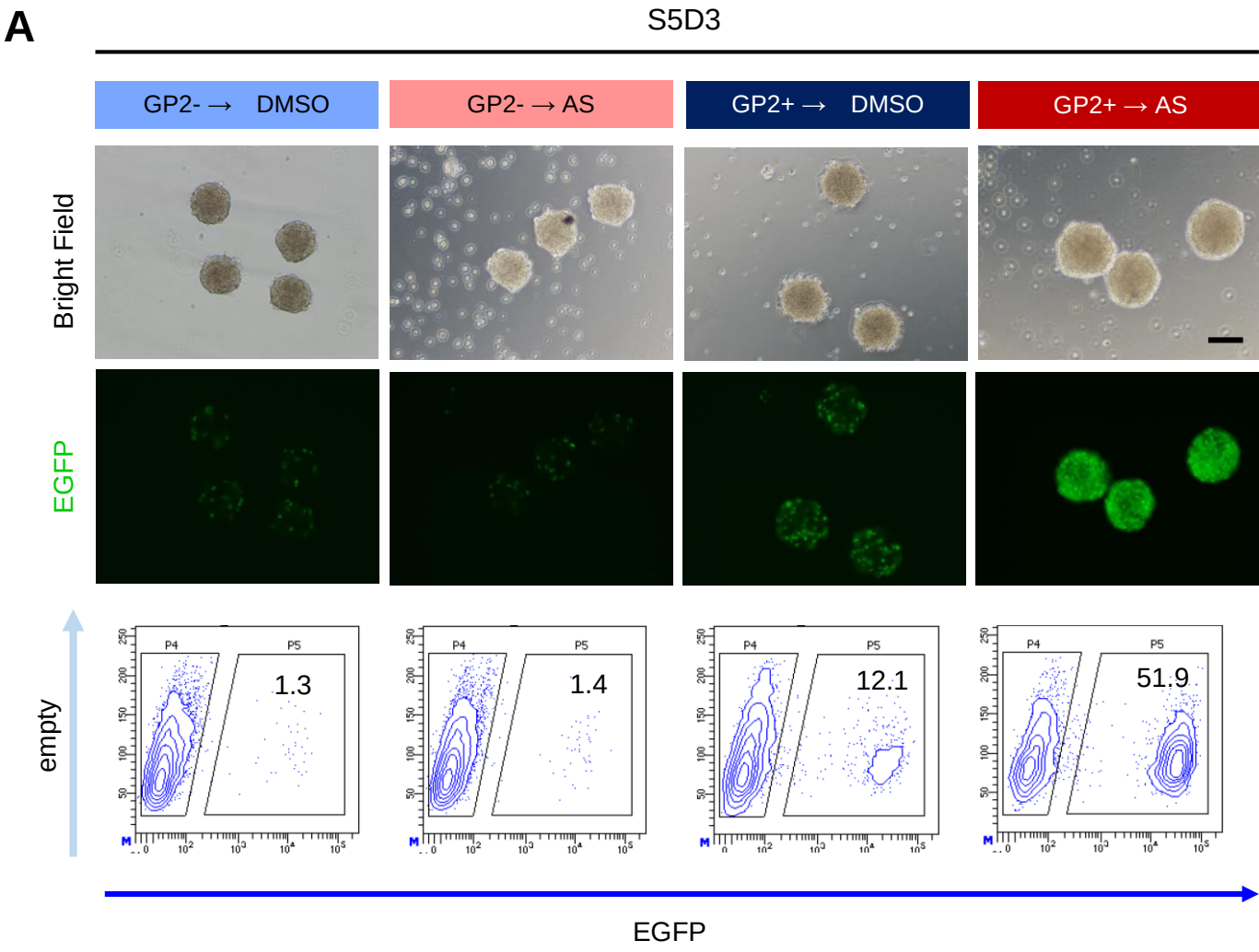


Supplemental Figure. 3



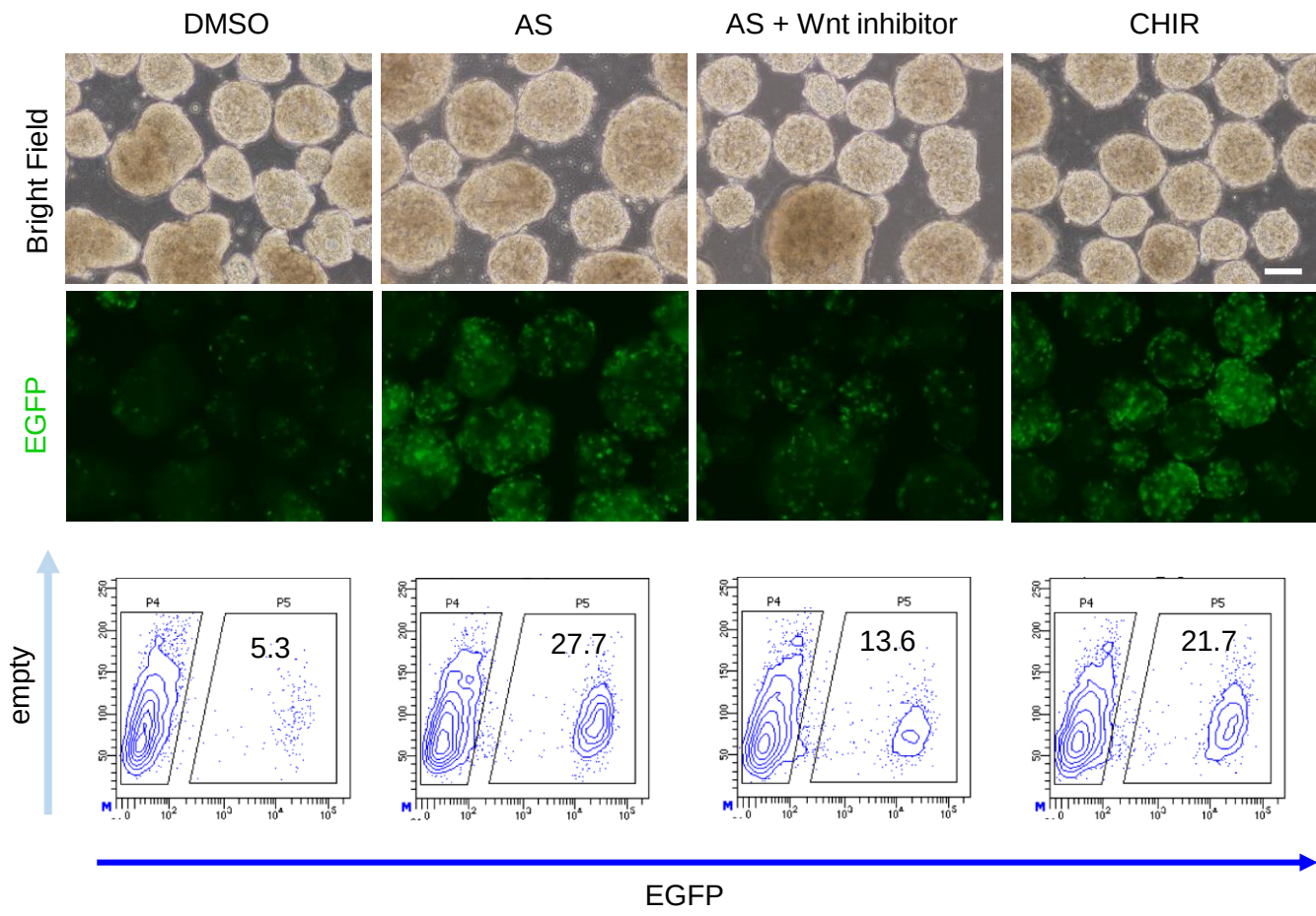
Supplemental Figure. 4



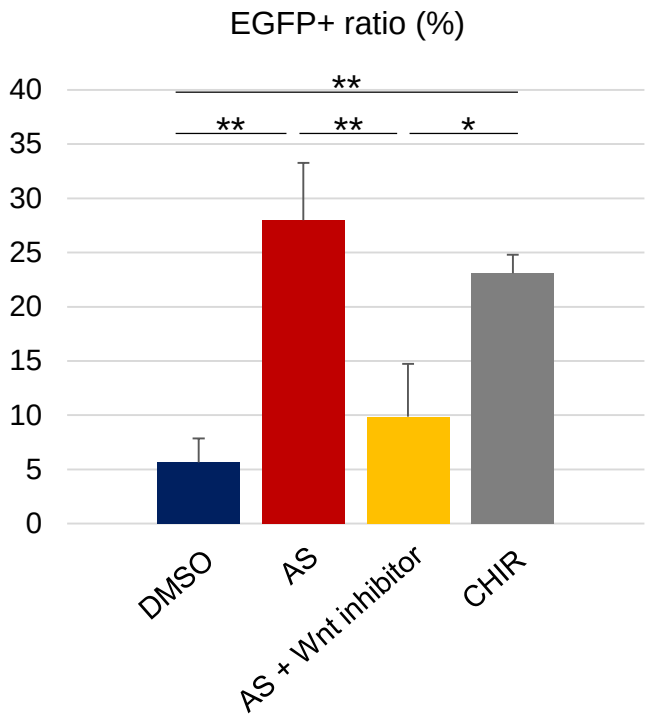


A

S5D3

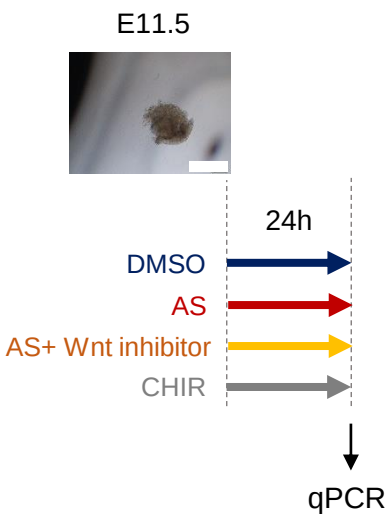


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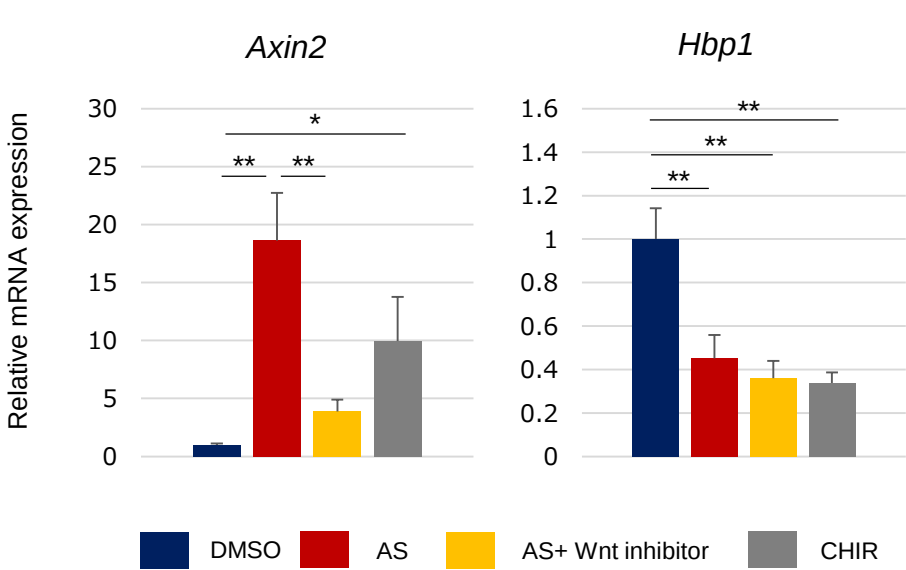


Supplemental Figure. 7

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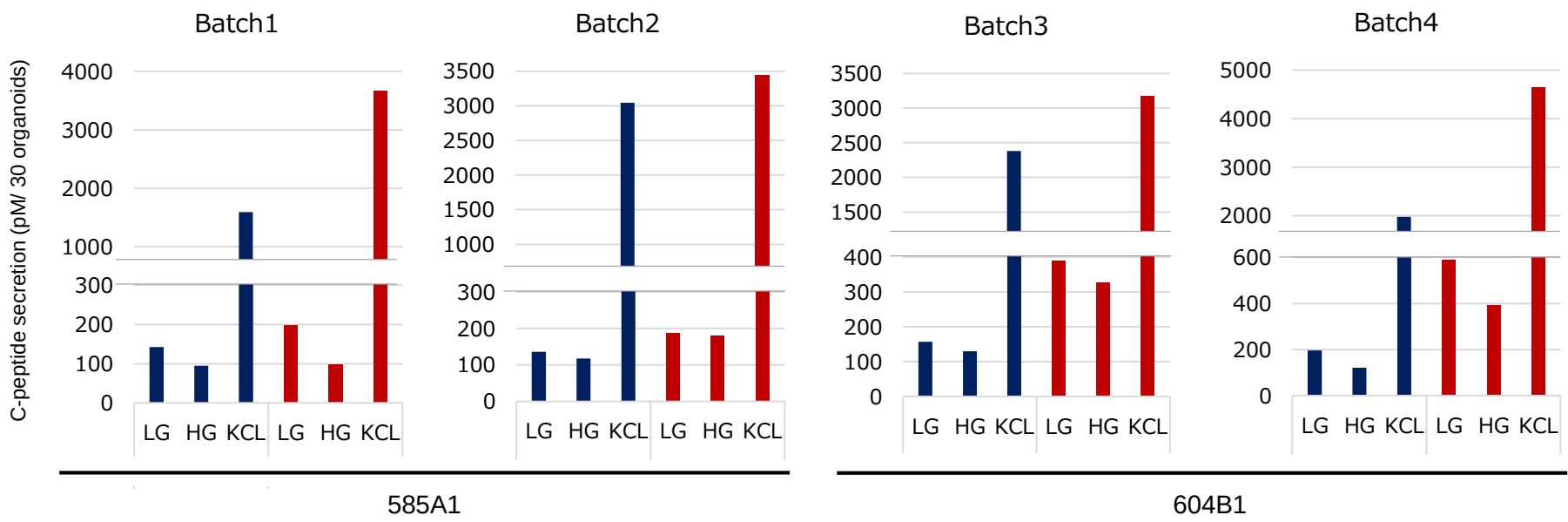


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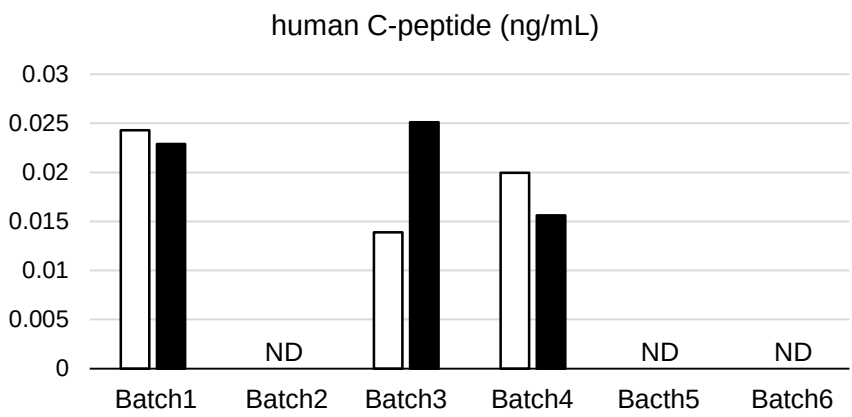


Supplemental Figure. 8

A



B



Supplemental Table 1.**Culture media contents**

Day	Basal media	Contents
S1D0	RPMI1640 with GlutaMAX (Thermo Fisher Scientific), 2% B27 (Thermo Fisher Scientific)	100 ng/mL activin A (R&D), 3 μ M CHIR99021 (CHIR; Wako), 100 nM wortmannin (Sigma), and 10 μ M Y-27632 (Wako)
S1D1	RPMI1640 with GlutaMAX, 2% B27	100 ng/mL activin A, 1 μ M CHIR
S1D2~	RPMI1640 with GlutaMAX, 2% B27	100 ng/mL activin A
S2D0~	RPMI1640 with GlutaMAX, 1.5% B27	50 ng/mL KGF (R&D), 2 μ M CHIR, and 100 nM LDN193189 (LDN; Sigma)
S3D0~	DMEM/F12 with GlutaMAX (Thermo Fisher Scientific), 1% B27	50 ng/mL KGF, 2 μ M retinoic acid (RA; Sigma), 100 nM LDN, 250nM SANT1 (Sigma), 250 μ M ascorbic acid (Sigma), 10 μ M Y27632, and 2.5 % Matrigel
S4D0	DMEM/F12 with GlutaMAX, 1% B27	25 ng/mL KGF, 100 nM RA, 100 nM LDN, 250 nM SANT1, 250 μ M ascorbic acid, and 200 nM ((2S,5S)-(E,E)-8-(5-(4-(trifluoromethyl) phenyl)-2,4-pentadienoylamino) benzolactam (TPB; PKC activator, Calbiochem)
S4D1	DMEM/F12 with GlutaMAX, 1% B27	S4D0 media + 10 μ M Y-27632
S4D2~	DMEM/F12 with GlutaMAX, 1% B27	50 ng/mL KGF, 1 mM nicotinamide (Wako), 100 nM RA, 100 nM LDN, 250 nM SANT1, 250 μ M ascorbic acid, 200 nM TPB, and 10 μ M Y27632
S5D0~	DMEM/F12 with GlutaMAX, 1% B27	1 mM nicotinamide (Wako), 100 nM RA, 100 nM LDN, 250 nM SANT1, 200 nM TPB, 10 μ M DAPT (Notch inhibitor, Sigma), 10 μ M ALK5 inhibitor (Wako), and 10 μ g/ml of heparin (Sigma)
S6D0~	DMEM/F12 with GlutaMAX, 1% B27	1 mM nicotinamide, 100 nM RA, 100 nM LDN, 250 nM SANT1, 50 nM TPB, 10 μ M ALK5 inhibitor, 10 μ g/ml of heparin, and 1 μ M 3,3',5-Triiodo-L-thyronine sodium salt (T3; Sigma)
S6D2~	DMEM/F12 with GlutaMAX, 1% B27	1 mM nicotinamide, 10 μ M ALK5 inhibitor, 10 μ g/ml of heparin, and 1 μ M T3

Supplemental Table 2.

Antibodies used in this study (IHC; Immunohistochemistry, FC; Flowcytometry)				
Primary	Company	Catalog Number	IHC	FC
SOX17	R&D	AF1924	1/400	1/200
FOXA2	Millipore	07-633	1/400	1/200
HNF1B	BD	612504	1/50	1/50
PDX1	R&D	AF2419	1/400	1/200
NKX6.1	DSHB	F55A12	1/100	1/50
NKX6.1	R&D	AF5857	no use	1/50
NGN3	R&D	AF3444	1/250	1/50
INSULIN	CST	3014	1/400	1/50
GULCAGON	Sigma	G2654	1/1000	1/250
SYNAPTOPHYSIN	NovusBiologicals	NBP1-22901	1/200	no use
TRYPsin	R&D	AF3586	1/200	no use
CK19	Dako	M088	1/200	no use
Secondary	Company	Catalog Numeber	IHC	FC
Alexa Fluor 488 Donkey-anti goat	Invitrogen	A11055	1/500	
Alexa Fluor 488 Donkey-anti sheep	Invitrogen	A11015		
Alexa Fluor 488 Donkey-anti rabbit	Invitrogen	A21206		
Alexa Fluor 568 Donkey-anti mouse	Invitrogen	A10037		
Alexa Fluor 647 Donkey-anti mouse	Millipore	AP192SA6		
Alexa Fluor 647 Donkey-anti goat	Millipore	AP180SA6		
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	ThermoFisher	D1306		
conjugated	Company	Catalog Numeber	condition	FC
mouse IgG1-PE (for isotype control)	MBL	M075-5	RT, 30min 2uL/ 1.0x10 ⁵ cells	
GP2-PE	MBL	D277-5		
isotype control IgG	Company	D277-5	IHC	FC
goat IgG	R&D	AB-108-C	isotype control IgG at the same concentration as the primary antibody	
rabbit IgG	R&D	AB-105-C		
sheep IgG	R&D	5-001-A		
mouse IgG1	R&D	MAB002		

Supplemental Table. 3

qPCR primer sequences

human	Forward	Reverse	Reference
<i>PDX1</i>	CCTTTCCCATGGATGAAGTC	CGTCCGCTTGTTCTCCTC	-
<i>NKX6.1</i>	ATTCGTTGGGGATGACAGAG	TGGGATCCAGAGGCTTATTG	Toyoda et al., 2015
<i>SOX9</i>	GAAGCTCGCGGACCAGTAC	GCTTCTCGCTCTCGTTCAGAAG	-
<i>NGN3</i>	CGCTGCTCATCGCTCTCTATTC	TCTCACGGGTCACCTGGAC	-
<i>GP2</i>	GCACTCCCTGGTGTAATCTGA	CTGAGGCTGCAAACCTGTGGA	Ameri et al., 2017
<i>HES1</i>	TCAACACGACACCGGATAAA	TCAGCTGGCTCAGACTTTCA	-
<i>AXIN2</i>	AGTGTGAGGTCCACGAAAC	TGGCTGGTGCAAAGACATAG	-
<i>TCF-1</i>	GGTGTCCTCCCAACTCTCTCT	GCCTGAGGTCAGGGAGTAGA	-
<i>LEF-1</i>	GACGAGATGATCCCTTCAA	ACAAACCTCTCAGGAGCCCT	-
<i>PDK4</i>	TGAGACTCGCCAACATTCTG	TCTGGTCATCTGGGCTTTTC	-
<i>HBP1</i>	GGACAAGAGAGCCTCAGGAA	CATGCCAGATTGGGTAGGAT	-
<i>NKX2.2</i>	TCTACGACAGCAGCGACAAC	TTGTCATTGTCCGGTGA CTC	-
<i>B2M</i>	GTGCTCGCGCTACTCTCTCT	TCAATGTCGGATGGATGAAA	-
mouse	Forward	Reverse	Reference
<i>Pdx1</i>	GAAATCCACCAAAGCTCACG	ACGGGTCCTCTTGTTTCCT	-
<i>Nkx6.1</i>	CCCGGAGTGATGCAGAGT	TTTGCTGTCCAGAGAACGTG	-
<i>Sox9</i>	AGGAAGCTGGCAGACCAGTA	TGTAATCGGGGTGGTCTTTC	-
<i>Axin2</i>	TGTGAGATCCACGAAACAG	TGGATAACTCGCTGTCGTTG	-
<i>Hbp1</i>	CCTGTGATGAGCACATGGAG	AGCCAGTCCACCTCATCTTC	-
<i>B2m</i>	TGGTAAAGCAAAGAGGCCTAA	AGAAGTAGCCACAGGGTTGG	-

Supplemental Table. 4

qPCR primers for copy number assays of EGFP reporter

	Forward	Reverse
<i>PGK*</i>	ATAGCAGCTTTGCTCCTTCG	CGGAGATGAGGAAGAGGAGA
<i>DLX5</i>	CCCCGTAGGGCTGTAGTAGT	TTCCAAGCTCCGTTCAGAC

Genotyping primer sequences for EGFP reporter

index**	sequence
P1	CTGAACTTGGCGACCAGAAG
P2	CTTGCTCACCATAGGTCCAG
P3	GGA CTCTGGGGTTCGAATAA
P4	CAGAAGTGCTCTTCCTCAGC
P5	ATGGTGAGCAAGGGCGAGGA

The site of the PGK* and index** primers (P1-P5) are shown in Supplemental Fig 4A.