Supplement

Supplemental Methods

Matrigel. In order to better optimize our system, we selected a single Matrigel lot for all experiments based on its ability to somewhat support our induction of our weakest iPSC lines; however, even with an optimal Matrigel lot without doxycycline, we did not see efficient differentiation in such lines.

Cell Culture of iPSCs. The media conditions to maintain human iPSCs have all been previously described (48). Cells were grown on growth factor reduced Matrigel Matrix basement membrane (REF 356231, lot 7114006, Corning). For passaging the cells Accutase (StemCell Technologies, Vancouver, BC) was used for 5 min at 37C and then stopped and pelleted by gentle centrifugation with iPSC media containing 10μM Y-27632 (Selleckchem, Houston, TX) (49). The dissociated iPSCs were seeded in new plates with media containing Y-27632, and after 24 hours replaced with media alone. IPSC media was changed daily. Cells were routinely tested and confirmed negative for Mycoplasma contamination by MycoAlert Detection Kit (Lonza #LT07-318).

Induction of Hepatic Definitive Endoderm (days 0-7). IPSCs were plated on 6, 12, or 96-well Matrigel-coated plates (growth factor reduced, REF 356231, lot 7114006, Corning) at different densities for differentiation. For example, typical plating density in a single well of a 6 well plate was 500,000 cells or 20,000 cells per well for 96 well plates. The differentiation process consisted of one week of RPMI media supplemented with Gem21 NeuroPlex without insulin (Gemini Bio-Products catalog #400-962, Gemini, West Sacramento, California), Glutagro catalog# 25-015-Cl (Corning, Manassas, VA), Non-essential amino acids catalog # 11-140-050 (Gibco), Sodium Butyrate (0.5 mM) catalog # B5887 (Sigma Aldrich) and recombinant human

Activin A (100 ng/mL) Peprotech catalog #120-14E for 7 days at 20% oxygen, 5% $C0_2$. On the day before differentiation (day 0), cells were re-plated on Matrigel coated plates in the presence of 2μM doxycycline and 10μM Y-27632. On day 1 of induction, 2% KnockOut Serum Replacement (KSR) Gibco Catalog # 10828028, CHIR-99021 Selleckchem catalog # S2924 (3mM), PI-103 (50nM) Selleckchem catalog # S1038 and recombinant human BMP4 (10ng/mL) Peprotech catalog #120-05ET and FGF2 (20ng/mL) Peprotech catalog #100-18B were added to the media. On day 2, 1% KSR, PI-103 (50nM) and BMP4 (10ng/mL) and FGF2 (20ng/mL) and on day 3, 0.2% KSR and PI-103 (50nM) were added to the media. Doxycycline hyclate (Acros Organics catalog #446061000) was added throughout definitive endoderm induction at various doses as described in text with a standard dose being 2μM.

Differentiation of Definitive Endoderm to hepatocytes (days 8-23). IPSC-derived human endoderm from above was cultured continuously on Matrigel or split up to 1:4 onto Matrigel plates for hepatic differentiation. Definitive endoderm (DE) was cultured in Iscove's modified Dulbecco's medium (Gibco catalog # supplemented with Gem21 NeuroPlex without insulin; Gemini Bio-Products catalog #400-962), Glutagro, NEAA, 0.3 mM monothioglycerol, 0.126 M/mL human insulin (Sigma), and 100 nM dexamethasone. To induce hepatoblasts, cells were treated with FGF2 (10 ng/mL) and BMP4 (20 ng/mL) for 5 days at 5% oxygen, 5% $C0_2$. To further differentiate the cells towards hepatocytes, cells were treated with FGF2 (10 ng/mL), BMP4 (20 ng/mL), and recombinant human HGF (20 ng/mL) Peprotech catalog #100-39 for 5 days at 5% oxygen, 5% $C0_2$. This was followed by changing the culture conditions to Lonza Hepatocyte Culture Media BulletKit (HCM Catalog # CC-3198) with HGF (20 ng/mL) and recombinant human Oncostatin M (20 ng/mL) Peprotech catalog # 300-10 for 5 additional days at 20% oxygen, 5% $C0_2$. In the BulletKit, use of EGF was excluded.

Differentiation to Pancreatic Progenitors Media and Details. The media formulation for each stage is as follows: Stage 1: 500mL MCDB-131 (Corning, 15-100-CV) + 0.22g glucose (MilliporeSigma, G7528) + 1.23g sodium bicarbonate (MilliporeSigma, S5761) + 0.022g vitamin C (MilliporeSigma, A4544) + 10g fatty-acid free bovine serum albumin (FAF-BSA) (Lampire Biological Laboratories, 7500812) + 10µL Insulin-Transferrin-Selenium-Ethanolamine (ITS-X) (Invitrogen, 51500056) + 5mL GlutaMAX (Invitrogen, 35050079) + 5mL Penicillin-Streptomycin (P/S) solution (Corning, 30-002-CI). Stage 2: 500mL MCDB-131 + 0.22g glucose + 0.615g sodium bicarbonate + 0.022g vitamin C + 10g FAF-BSA + 10µL ITS-X + 5 mL GlutaMAX + 5mL P/S. Stage 3, 4: 500 mL MCDB-131 + 0.22 g glucose + 0.615 g sodium bicarbonate + 0.022 g vitamin C + 10g FAF-BSA + 2.5mL ITS-X + 5mL GlutaMAX + 5mL P/S. HUES8 cells were grown and seeded in mTeSR-1 media (STEMCELL Technologies) with 10 µM Rock inhibitor Y-27632 (STEMCELL Technologies) for induction to endoderm.s

Samples were taken for QC at Pluripotency, End Stage 1, End Stage 3, and End Stage 4. Samples for immunofluorescence were dissociated with TrypLE Express (Gibco, 12604013), seeded on 96-well plates coated with Growth Factor Reduced Matrigel (Corning, 354230), and fixed in 4% PFA for 15 minutes after overnight incubation. Samples for flow cytometry were dissociated with Accumax (Innovative Cell Technologies, AM105) and fixed in 4% PFA for 15 minutes.

Western blot analysis. Performed as previously described (49). Protein bands were visualized by Odyssey Infrared Imager scanner and band intensities were quantified using the Image Studio software (LI-COR Biosciences).

DNA Primers. Primers were made by IDT. Primers used for qrtPCR include cMyc-F 5'-AAACACAAACTTGAACAGCTAC-3', cMyc-R 5'- ATTTGAGGCAGTTTACATTATGG-3', BCL2L1-F 5'-TCCTTGTCTACGCTTTCCACG-3', BCL2L1-R 5'-GGTCGCATTGTGGCCTTT-3', GAPDH-F 5'-AGCCACATCGCTCAGACAC-3', GAPDH-R 5'-GCCCAATACGACCAAATCC-3'. ALB-F 5'- GCCTTTGCTCAGTATCTT-3', ALB-R 5'-AGGTTTGGGTTGTCATCT-3', A1AT-F 5'-AGACCCTTTGAAGTCAAGCGACC-3', A1AT-R 5'-CCATTGCTGAAGACCTTAGTGATGC-3', HNF4alpha-F 5'- CATGGCCAAGATTGACAACCT-3', HNF4alpha-R 5'-TTCCCATATGTTCCTGCATCAG-3', FOXA2- F 5'- CGGGCTCCATGAACATGTCG-3', FOXA2-R 5'-GCGAGATGTACGAGTAGGGC-3', CYP3A4-F 5'-TGTGCCTGAGAACACCAGAG-3', CYP3A4-R 5'-GTGGTGGAAATAGTCCCGTG-3', AFP-F 5'-AGAACCTGTCACAAGCTGTG-3', AFP-R 5'-TGGTAGCCAGGTCAGCTAAA-3'

FACS analysis of cells. After lentiviral infection by the Doxycycline-inducible cMYC or Doxycycline-inducible (BCL-XL) vectors, cells were separated on a FACS AriaII (BD Biosciences) for GFP positive cells. Individual clones were isolated by sorting single iPSCs to wells of a 96-well plate precoated with Matrigel with iPSC media containing 10μM Y-27632 and 2μM doxycycline. Clonal iPSC isolates were expanded by weekly passaging and standard iPSC culture as described above. For FACS analysis of endoderm, single-cell suspensions of iPSCs and iPSC-derived endoderm were permeabilized with 0.1% triton X-100 (Sigma, CITY), and then incubated with one of the conjugated antibodies CXCR4-PE (Biolegend 306505), or FOXA2 Alexa Fluor 700-conjugated antibody (R&D Systems; FAB24001N), or SOX17 Alexa Fluor 488 conjugated antibody (R&D Systems; clone 614013). Analysis was performed on a FACS AriaII and FlowJo software (FlowJo LLC, BD Biosciences) using iPSCs as negative controls.

Immunohistochemistry and Cell staining IPSCs and iHeps were fixed using 4% paraformaldehyde for 15 min, permeabilized using 0.1% triton X-100 for 10 minutes followed by 3x PBS (phosphate buffered saline, Corning Life Sciences) rinses and blocked with 1.5% bovine serum albumin for 1 h. Samples were incubated with primary antibody overnight (List of all antibodies with dilutions are shown in Supplementary Table 4). Cells incubated in primary antibody in blocking solution at the stated dilutions. The cells were rinsed extensively with PBS and incubated for 60 min with secondary antibody at 1:1000 in blocking solution. After washing 3 times with PBS, DAPI (4′,6-diamidino-2-phenylindole, MP Biomedicals, Solon, OH) was added at 300 nM for 15 min. After 3x PBS washes, the cells were imaged using an BIOTEK Cytation 5 Microscope (Winooski, VT).. Cells were then fixed with 4% PFA and Hoechst 33342, rinsed in PBS and imaged with Cytation5 imager, BioTek Instruments. Images were analyzed using Biotek Imaging software. Primary and secondary mask analyses on digital images were used to quantify based on nuclei and antibody staining in secondary mask. Threshold analysis was set to exclude artifacts.

Quantification of Cell Confluence Cell confluence was calculated with the BioTek Cytation5 imaging reader. Cells were fixed with 4% PFA, rinsed with PBS, and imaged in the BioTek Cytation5 imaging reader using brightfield. Using a fine-tuned Autofocus, allowing the system to focus on a known target repeatedly, images were captured in experimental mode for uniformity then analyzed with Gen5 Image Prime 3.08 automated “cell confluence” feature. The settings used were: Detection Method-Imaging; Read Type-Endpoint; Process Mode-Well Mode; Color Channel-Bright Field; Exposure-LED5, Integration time:50, Gain: 0; Autofocus Options-Autofocus with optional scan; Objective-Zeiss 4x/10x.

Endoderm and iHep Differentiation and other Phenotypes were analyzed using various assays per manufacturer’s standard protocol including Albumin ELISA (Bethyl #E88-129), P450-Gla

CYP3A4 Assay (Promega V8801), Indocyanine Green (MP Biomedicals #215502005), BCA Protein Assay Kit (Fisher Scientific #0023227), Tetramethylrhodamine (TMRM Promega T5428), Mitotracker Green FM (Cell Signaling #9074), Caspase-Glo 8 Assay (Promega #G8201), and Caspase-Glo 9 Assay (Promega #G8211).

Supplementary Figure Titles and Legends

Supplementary Figure 1 Standard differentiation protocol

(A) Our old protocol for induction of iPSC to endoderm and iPSC-Heps.

(B) Induction of iPSC with doxycycline-inducible cMYC results in iPSC-Heps with appropriate staining of markers including A1AT (serpin family A member 1), AFP (alpha fetoprotein), Albumin, HNF4α (hepatocytte nuclear factor 4 alpha) and FOXA2 (n=3 technical replicates for three cell lines).

(C) Plating density versus confluency after 6 days of induction (n=3 technical replicates in 5 different cell lines, line AS7192 was tested in two different subclones designated -9 and -7). Confluency grade analyzed visually and defined as 1-no cells remain, 2-few cells, 3-about 75% covered well, 4-complete monolayer.

(D) Quantitative PCR for mRNA expression of cMYC relative to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in lentivirally transduced AG06103A-3 with and without doxycycline induction (n=3 technical replicates for each cell line).

Data were analyzed by Student's t test and presented as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$.

Supplementary Figure 2 Induction with Doxycycline and Dose Response.

(A) Comparison of control versus 1µM doxycycline (+Dox) induction over six different cell lines shows improved confluence and survival of cells (n=4 technical replicates per line).

(B) Titration of doxycycline showed that there was optimal level of doxycycline and a toxic level (n=4 technical replicates in three different cell lines, two shown here and one shown in **Figure 4B**).

(C) Flow cytometry analysis was used to determine the percentage SOX17, CXCR4, and FOXA2 expression during in iPSC-derived endoderm (n=3 technical replicates: iPSC 1023-5 shown).

Data were analyzed by Student's t test, error bars represent 95% confidence interval.

Supplementary Figure 3 Doxycycline and Mitochondria

(A) Immunofluorescence of iPSC line AS7192-9 two days after induction with staining for Hoechst 33342, Ki-67, Cleaved Caspase 3 (CC3), and combined showed greatly decreased CC3 in the presence of doxycycline (n=3 biologic replicates). At this time point, there is a slight decrease in Ki-67. First two rows are 10x with scale bar at 400µm and bottom two rows 20x with 200µm scale bar.

(B) Mitotracker Green analysis of 72-hour induced Endoderm quantifications for three cell lines. Treatment with low-dose doxycycline increases Mitotracker green staining (n=6 technical replicates in three lines).

(C) Mitotracker Green analysis of 24-hour induced Endoderm and comparison of other compounds affecting mitochondria including Doxycycline, Ruthenium Red, KB-R7943, and Gentamycin. Data was quantified for three cell lines. There were no significant differences when all four compounds across three cell lines are compared as a whole (n=3 technical replicates).

(D) Mitotracker Green immunofluorescence of 24-hour induced Endoderm (scale bar = 300µM) for compounds affecting mitochondria including Doxycycline, Ruthenium Red, KB-R7943, and Gentamycin for three cell lines (n=4 technical replicates).

(E) Immunohistochemical staining for FOXA2 of 24-hour induced Endoderm (scale bar = 300μM) for compounds affecting mitochondria including Doxycycline, Ruthenium Red, KB-R7943, and Gentamycin for two cell lines (n= 1 technical replicate).

(F) Assay for Caspase 9 levels for endoderm at 24 hours induction at low-dose titrated levels of doxycycline across three cell lines (n=4 technical replicates).

Data were analyzed by Student's t test and presented as mean ± SD. * $p < 0.05$; ** $p < 0.01$.

Supplementary Figure 4

(A) Induction of iPSC line AS7192 to endoderm was performed. Doxycycline was compared to tetracycline derivatives for the ability to rescue endoderm induction. Minocycline, and Methacycline showed similar ability to rescue endoderm at the 1 μM concentration with some toxicity at the 10μM concentration. Demeclocycline showed a similar trend. The number of cells is shown measured seven days after induction compared to the plating density at day 0 (n=3 technical replicates).

(B) Other antibiotics and other compounds affecting the mitochondria were compared to doxycycline. While most antibiotics failed to have the same affect, surprisingly Gentamycin was shown to have a strong increasing dose response. The number of cells is shown measured seven days after induction compared to the plating density at day 0 (n=3 technical replicates).

(C) Fluorescence of cells after Cardiogreen 30 minutes prior (upper figures), and 1 hour later in lower figures as Cardiogreen is secreted out and washed away (n=1 technical replicate across two cell lines).

(D) CYP3A4 Activity assay on cells shows no statistical difference in multiple wells. Four cell lines shown. Two cell lines failed to induce and differentiate without the presence of doxycycline (n=12 technical replicates).

(E) Immunohistochemical staining for ASGPR1 (asialoglycoprotein receptor 1) of terminally differentiated iPSC-Heps (scale bar = 300μM) with and without doxycycline (2μM) exposure during endoderm induction in three cell lines merged with Hoechst staining (n=2 technical replicates).

Data analyzed by two way ANOVA or Student's t test and presented as mean ± SD. * $p < 0.05$; ** $p < 0.01$.

Supplementary Figure 5

(A) Paired differentiation of iPSC Lines to endoderm and iPSC-Hep. Without doxycycline a number of cell lines underwent apoptosis before successful endoderm or did not produce enough cells at the iPSC-Hep stage. Two separate differentiations of AS7192-9 were produced at the iPSC-Hep stage (designated by *). Some iPSC lines failed validation for RNA Sequencing and were excluded in the RNA Sequencing data.

(B) Doxycycline (2μM) increases the survival of cells during FACS sorting especially for sorting of single cells to create clonal lines (n=3 technical replicates).

(C) Testing Effects of Doxycycline on Differentiation to Pancreatic lineage. The first panel shows immunohistochemistry for SOX17 and FOXA2 at the end of Stage 1:definitive endoderm. The second panel shows immunohistochemistry for PDX1 and CDX2 (caudal type homeobox 1) at the end of Stage 3: Pancreatic Progenitor 1. The third panel shows immunohistochemistry for CHGA1(chromogranin A) at the end of Stage 4: Pancreatic Progenitor 2 (n=1 technical replicate).

(D) Induction of iPSCs to endoderm were performed with reagents excluded to test requirement of different reagents (n=3 technical replicates for each of the 3 cell lines).

(E) Flow Cytometry analysis of Pancreatic differentiation to Stage 1 Definitive Endoderm and Stage 3 Pancreatic Progenitor 1 (vehicle control versus 2μM Doxycycline) (n=4 technical replicates).

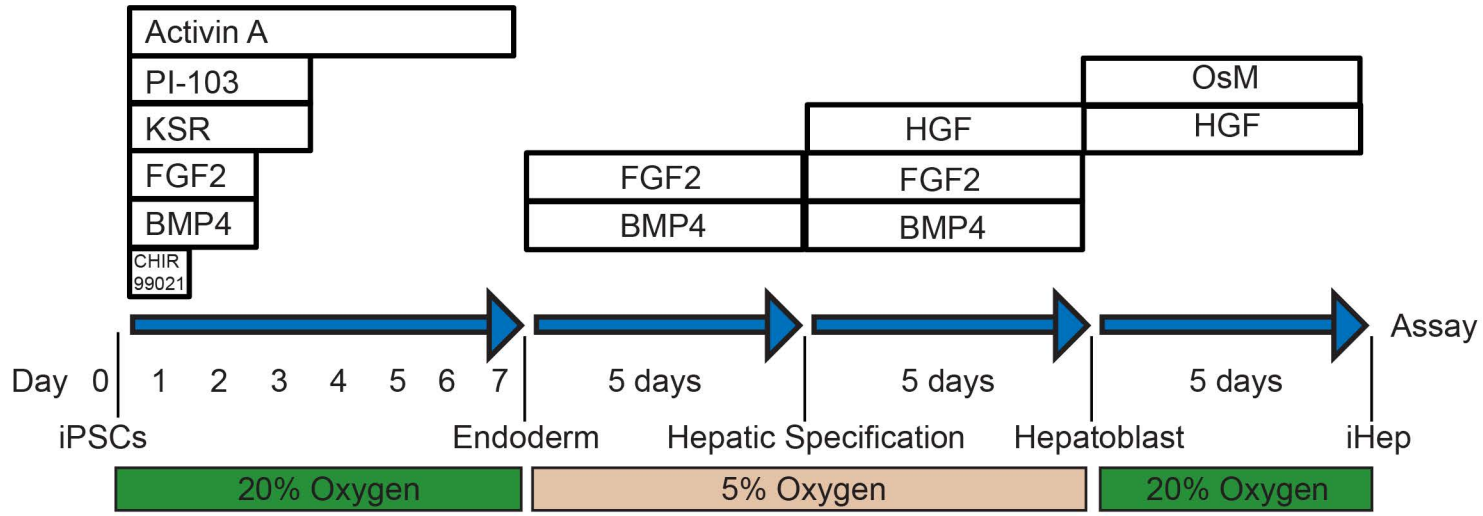
(F) Assessment of differentiation efficiency at each stage for cells undergoing differentiation to the Pancreatic Lineage (vehicle control versus 2μM Doxycycline during stage 1).

(G) Percentage well saturation during induction of iPSCs with vehicle (blue line), Y-27632 (red line), Doxycycline 2μM (green line), or Doxycycline plus Y27632 (n=3 technical replicates for each cell line). The bars show the statistical 95% confidence intervals.

Data were analyzed by two way ANOVA or Student's T test and presented as mean ± SD. * $p < 0.05$; ** $p < 0.01$. Error bars represent 95% confidence interval.

Supplementary Figure 1

A.



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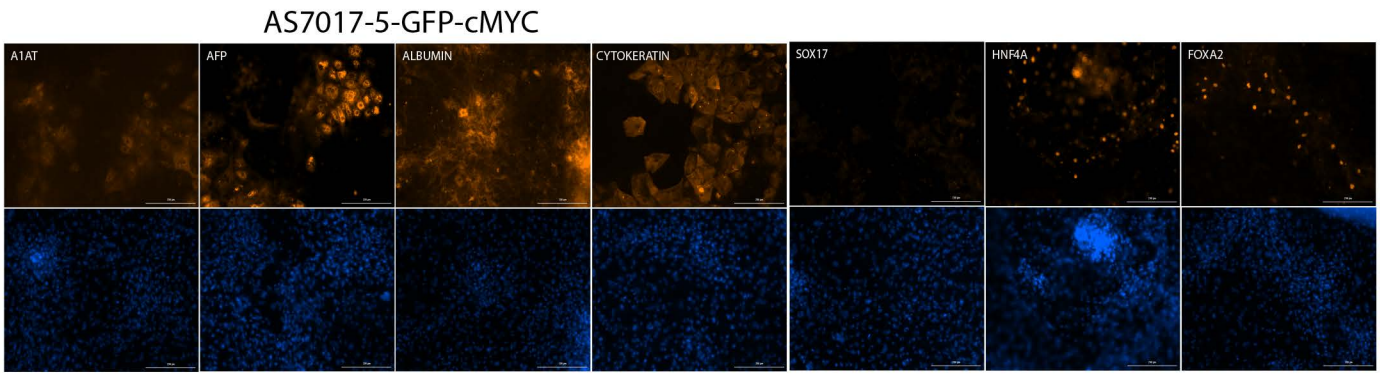

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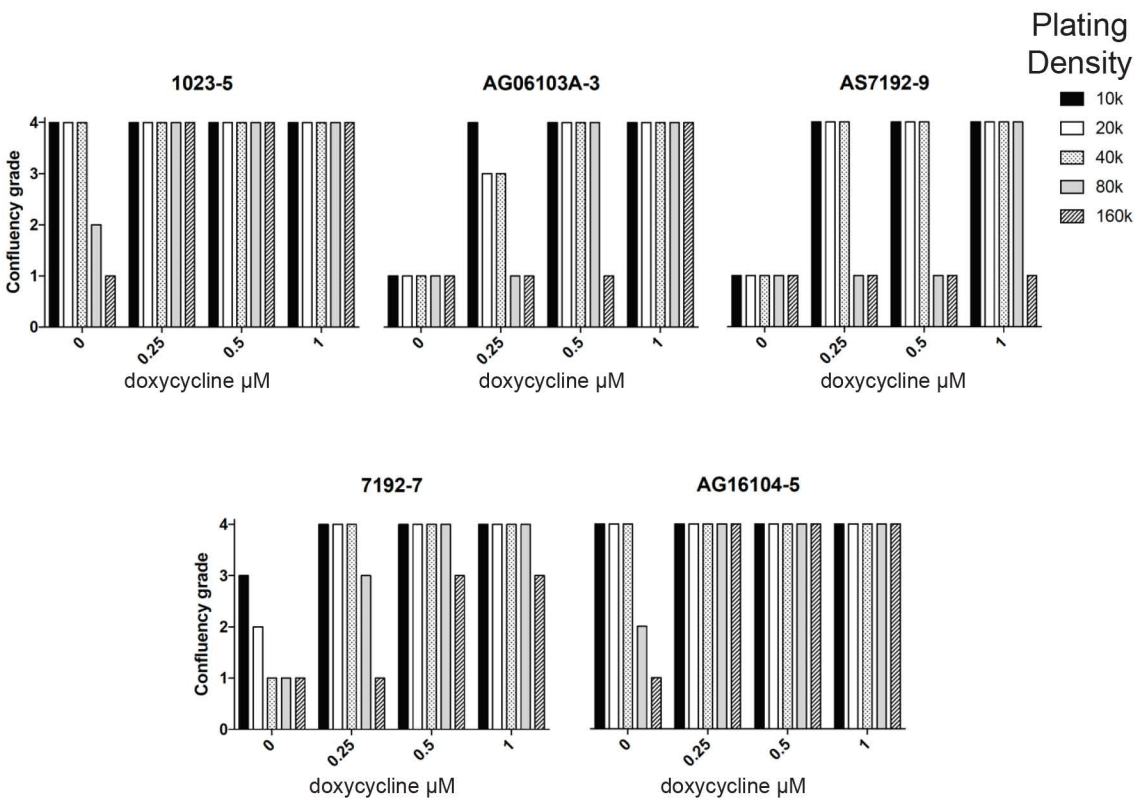

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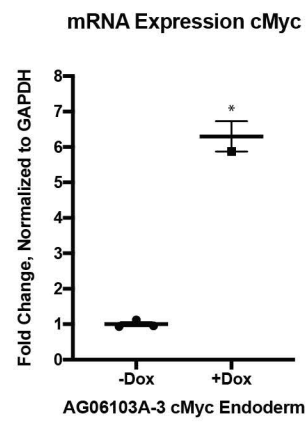
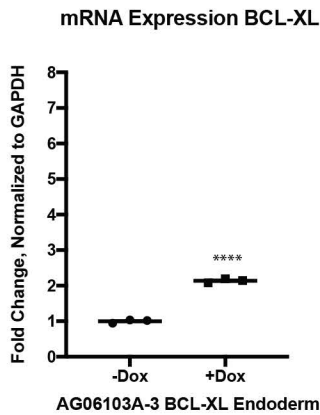
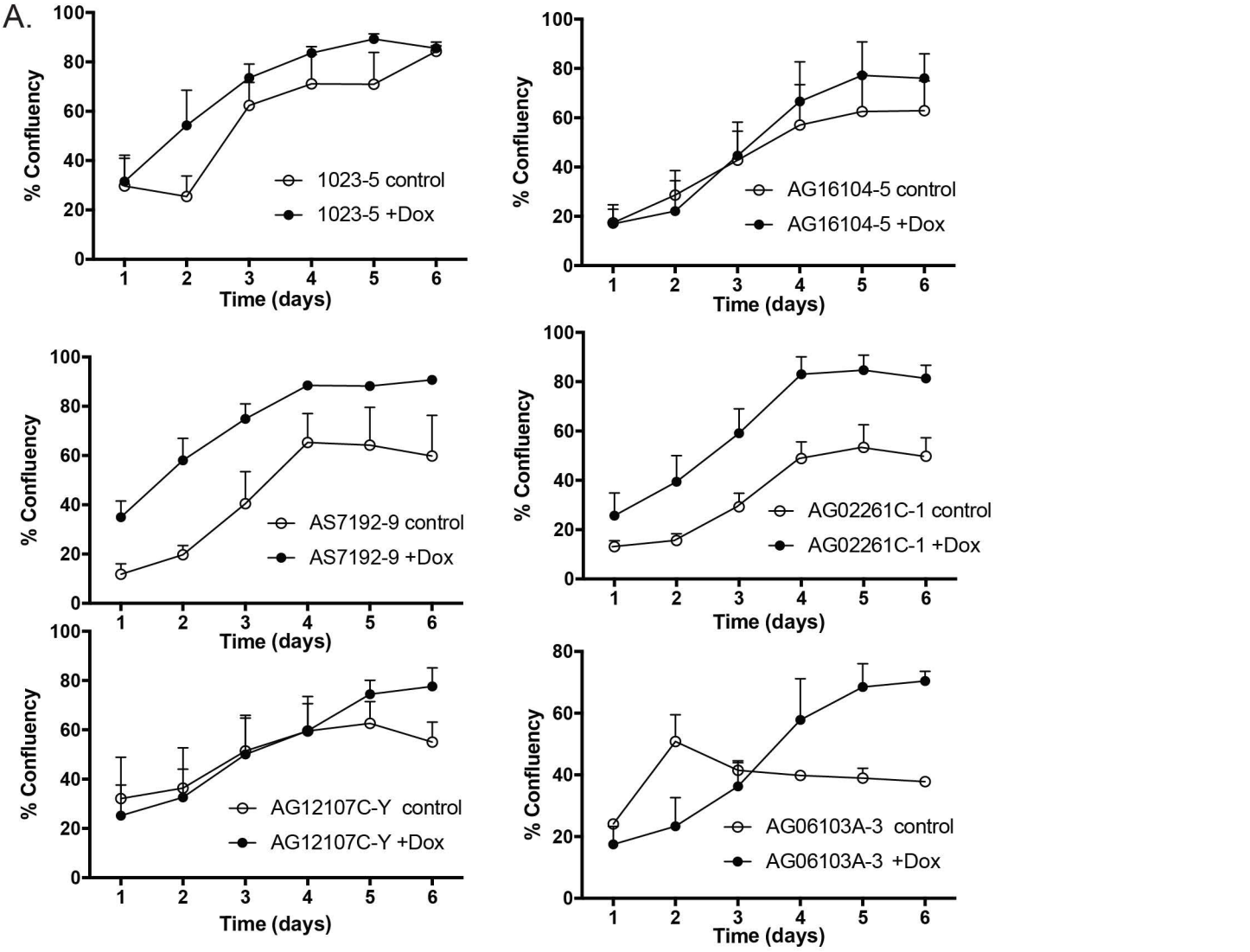
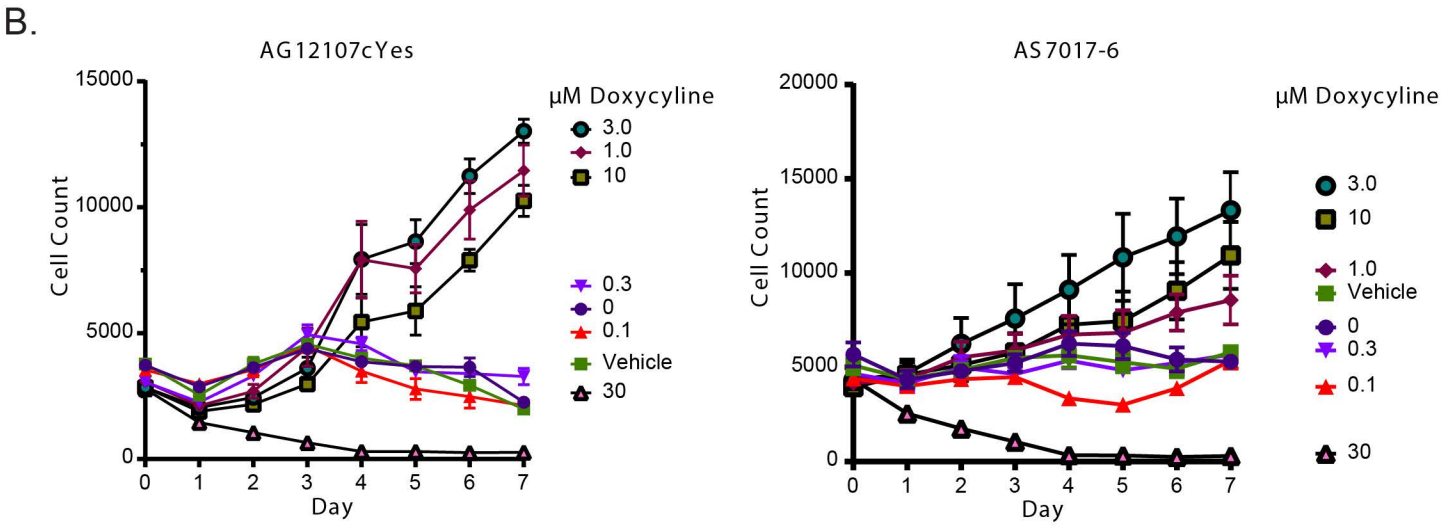
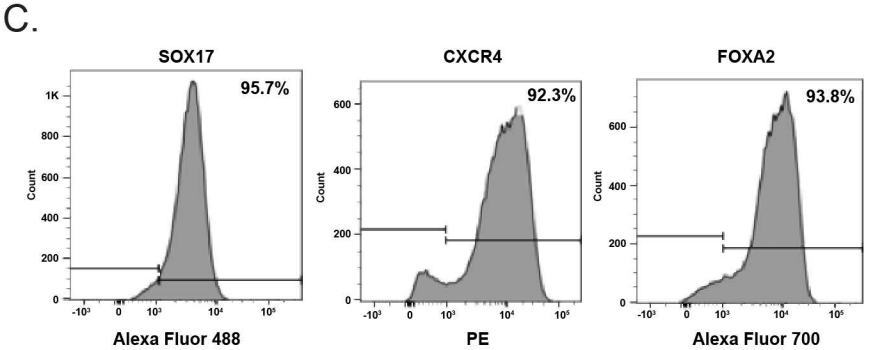




Supplementary Figure 2







Supplementary Figure 3

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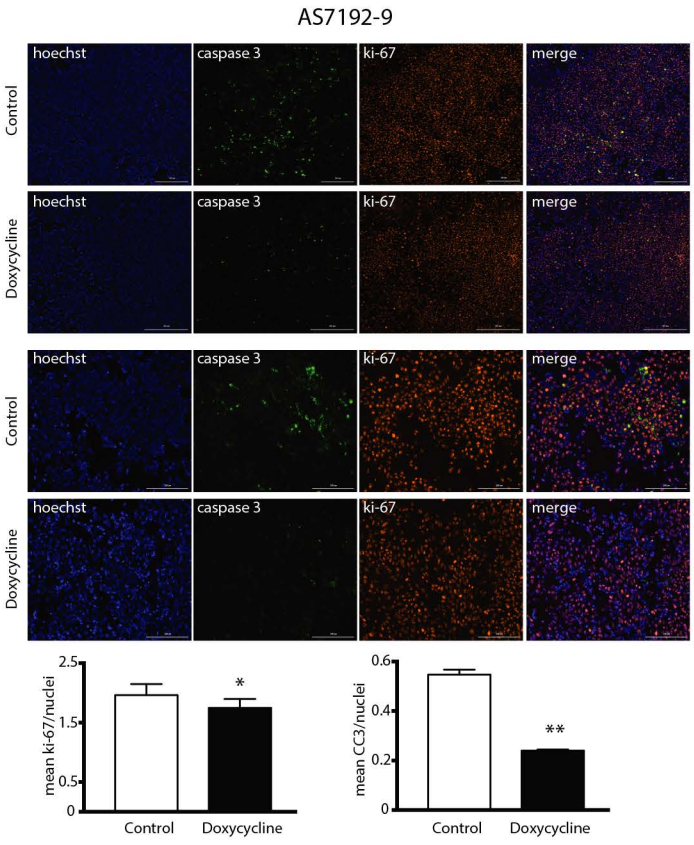

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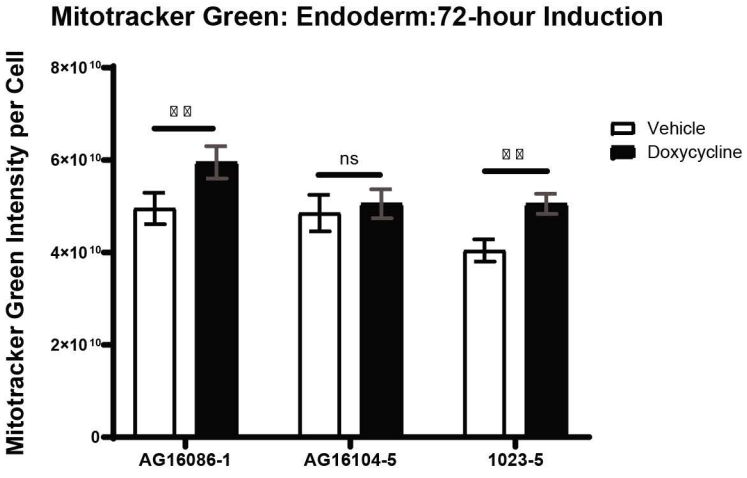

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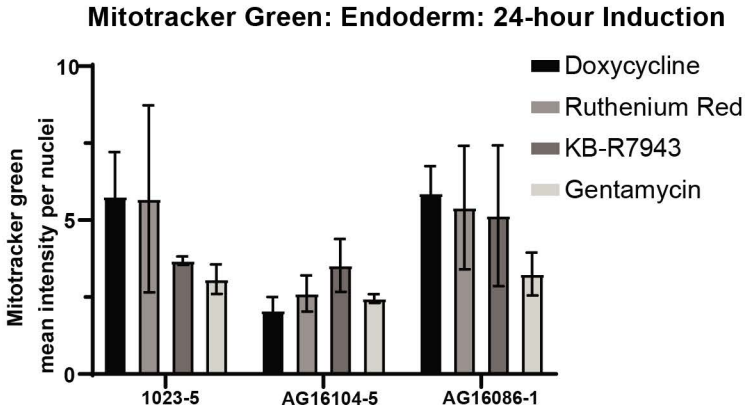

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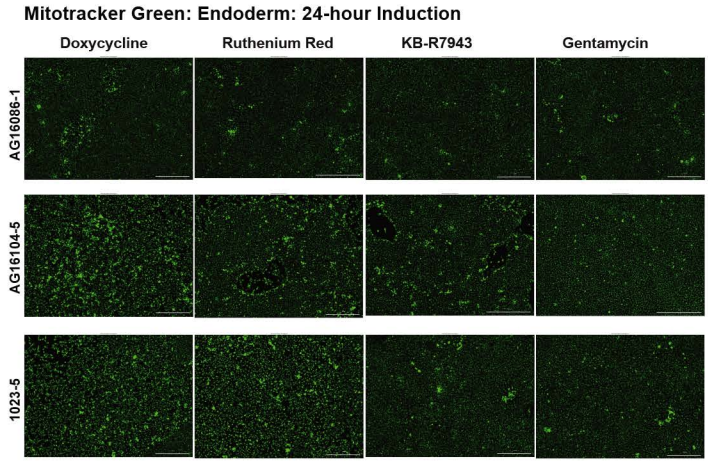

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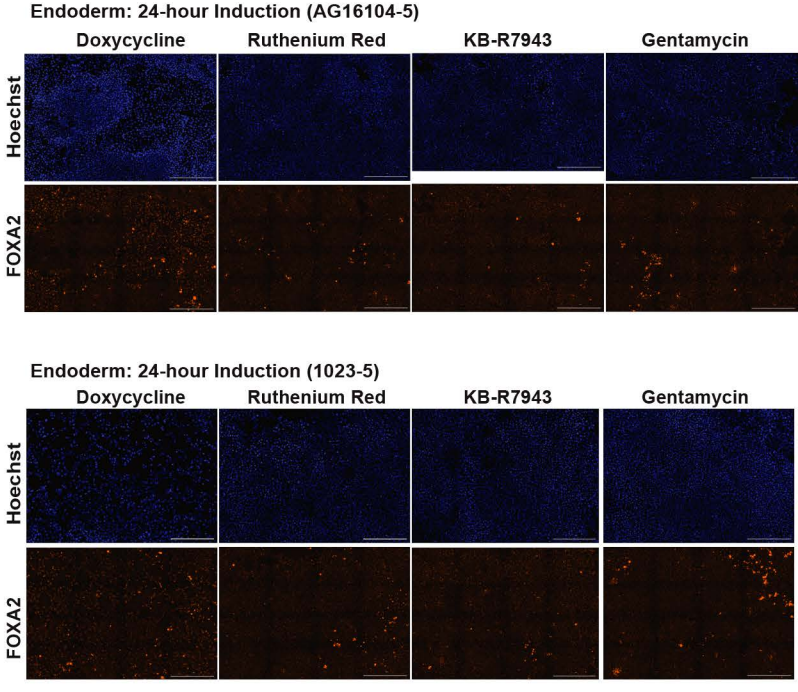

Supplementary Figure 4:

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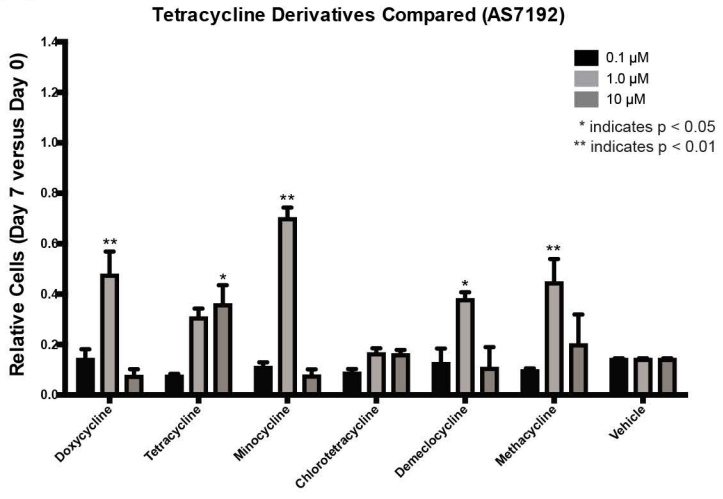

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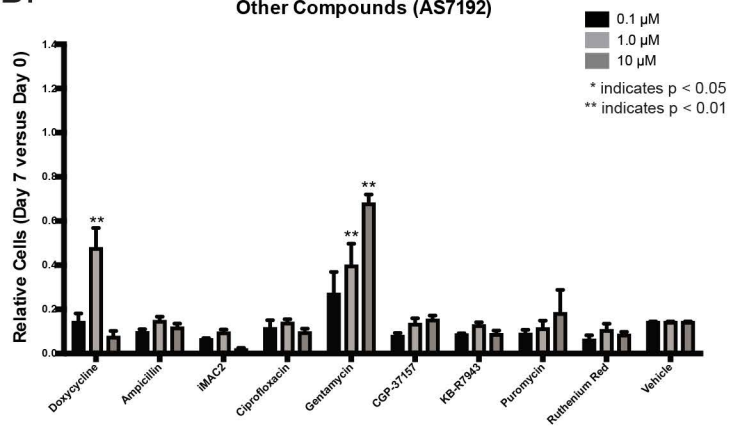

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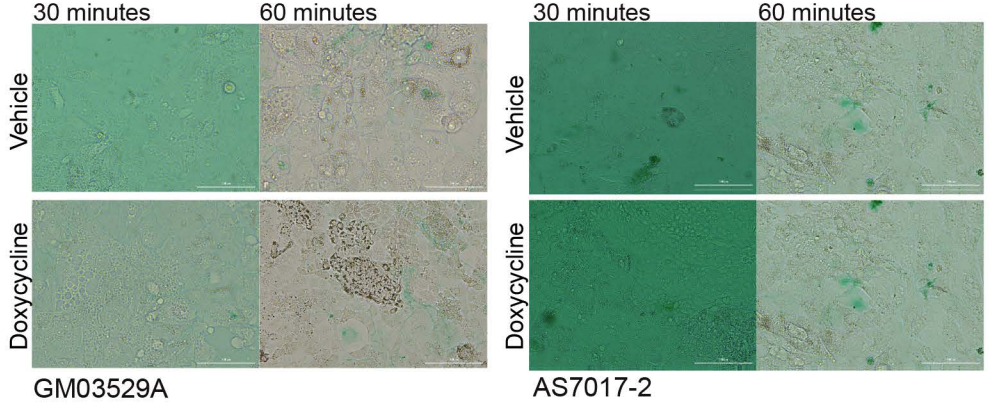

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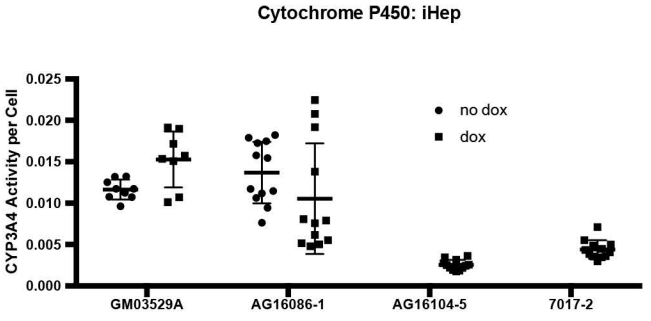
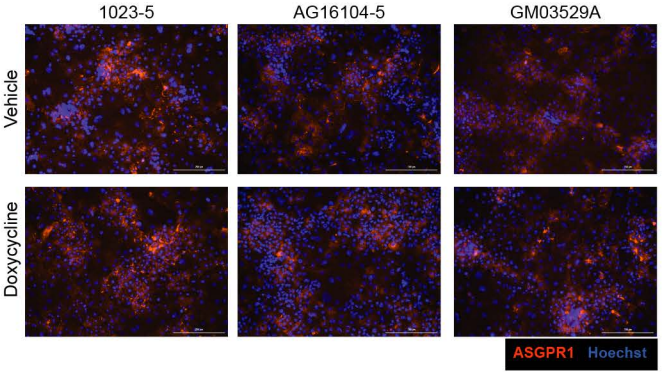


*AS7017-2 and AG16104-5 iHeps coulld not be generated without doxycycline

E.

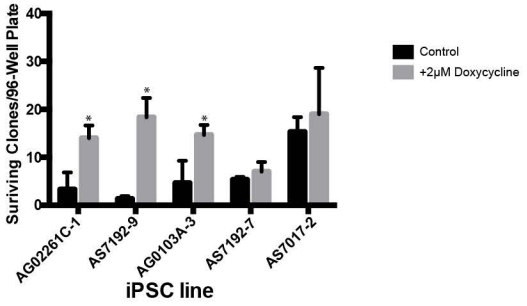


Supplementary Figure 5

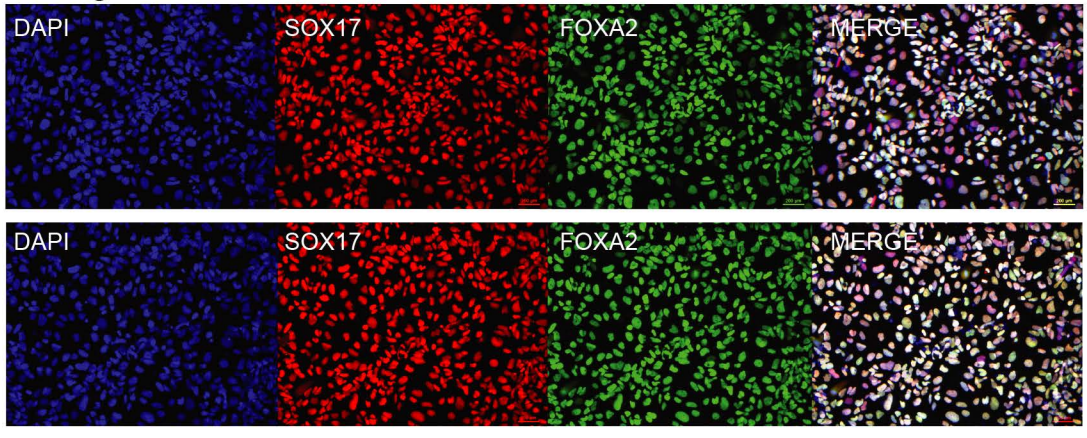
A. Paired Differentiation of iPSC Lines to Endoderm and iPSC-Heps

	iPSC Line	Vehicle endoderm	Dox endoderm	Vehicle iHep	Dox iPSC-Hep
1	AG16104-5	YES	YES	YES	YES
2	AG12107C-Y	FAILED	YES	YES	YES
3	AS4484-12	FAILED	YES	FAILED	YES
4	AS7192-9*	YES	YES	YES	YES
5	AS7017-6	YES	YES	FAILED	YES
6	AS7192-7	YES	YES	FAILED	YES
7	AG02261C-1	YES	YES	FAILED	YES
8	1023-5	FAILED	YES	YES	YES

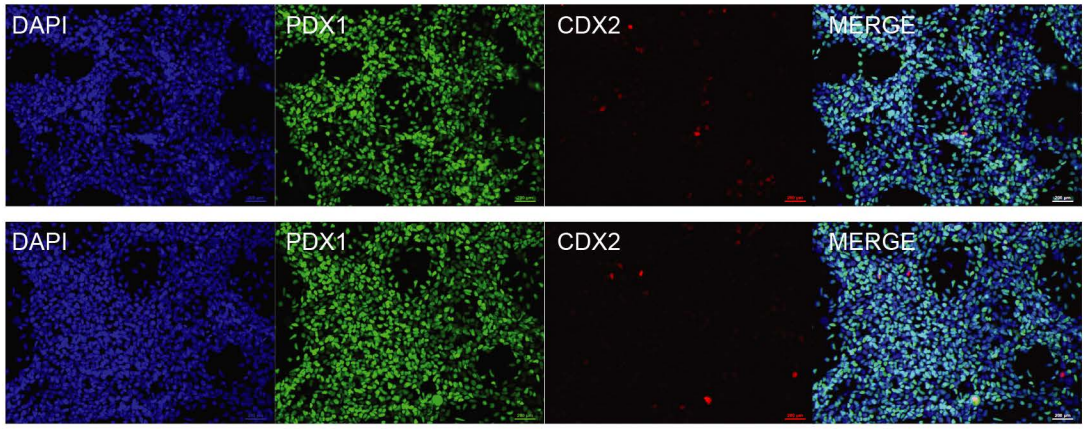
B. Doxycycline Increases Survival of cells during FACS



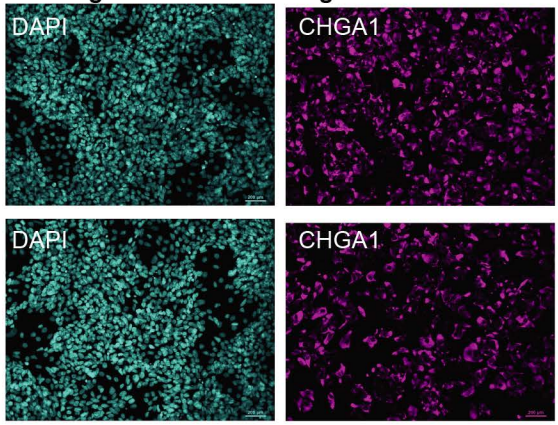
C. End Stage 1: Definitive Endoderm



End Stage 3: Pancreatic Progenitor 1



End Stage 4: Pancreatic Progenitor 2



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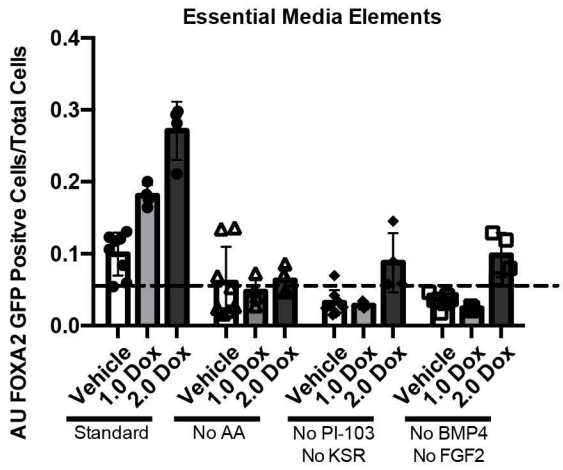
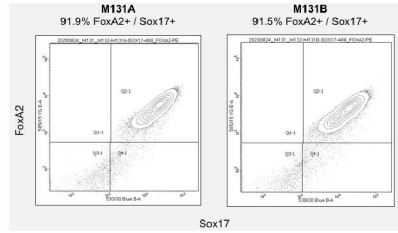
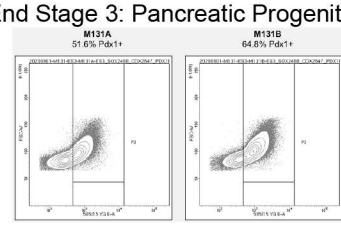


E. End Stage 1: Definitive Endoderm



End Stage 3: Pancreatic Progenitor 1



F.

M131 Control

QC stage	Population % by flow cyto.
Pluripotency	94.3% Oct3-4+/Nanog+
End Stage 1	91.9% FoxA2+/Sox17+
End Stage 3	56.3% Pdx1+ (avg, n=4)
End Stage 4	20.1% Nkx6.1+/Pdx1+ (avg, n=4) 34.7% CgA+ (avg, n=3)

M131 + 2μM Dox in Stage 1

QC stage	Population % by flow cyto.
Pluripotency	94.3% Oct3-4+/Nanog+
End Stage 1	91.5% FoxA2+/Sox17+
End Stage 3	67.6% Pdx1+ (avg, n=4)
End Stage 4	21.8% Nkx6.1+/Pdx1+ (avg, n=4) 31.7% CgA+ (avg, n=3)

G.