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TITLE:

Hepatic Progenitor Specification from Pluripotent Stem Cells Using a Defined Differentiation System

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KEYWORDS:

pluripotent stem cell, directed differentiation, hepatic progenitor, definitive endoderm, process automation, scale-up and standardization

SUMMARY:

The goal of this article is to provide a standardized approach to induce human hepatic progenitor differentiation from pluripotent stem cells. The development of this procedure with ready-to-use media formulations offer the user a facile system to generate human liver cells for biomedical research and translation.

ABSTRACT:

Liver disease is an escalating global health issue. While liver transplantation is an effective mode of therapy, patient mortality has increased due to shortages in donor organ availability. Organ scarcity also affects the routine supply of human hepatocytes for basic research and the clinic. Therefore, the development of renewable sources of human liver progenitor cells is desirable and is the goal of this study. To be able to effectively generate and deploy human liver progenitors on a large scale, a reproducible hepatic progenitor differentiation system was developed that can be used with both human embryonic and induced pluripotent stem cell lines. This protocol aids experimental reproducibility between users in a range of cell cultureware formats. These are important advantages over current differentiation systems

that will enhance the basic research and may pave the way towards clinical product development.

INTRODUCTION:

Liver disease represents a global health challenge, causing approximately 2 million deaths per year worldwide[1]. Although a number of model systems exist to study hepatic diseases and intervene clinically, the routine use of cell-based systems is limited by significant drawbacks (for a review see Szkolnicka et al.[2]). Advanced human pluripotent stem cell (hPSC) culture and somatic cell differentiation methods represent promising technologies to develop tools for basic biomedical research and renewable sources of pluripotent cells for the clinic[3,4].

To date, multiple protocols for hepatocyte-like cell (HLC) differentiation have been developed[5–8]. These protocols attempt to recreate aspects of human liver development by using a combination of small molecules and growth factors[9,10]. Most protocols consist of a stepwise differentiation process, where hPSCs are primed to definitive endoderm, followed by hepatic progenitor specification[11–13], and ending with HLC specification. HLCs produced by these protocols display a mixture of fetal and adult phenotypes. This includes the expression of alpha fetoprotein (AFP), hepatocyte markers HNF4α, albumin (ALB), as well as drug metabolizing capacity[14–16]. Between laboratories, HLC differentiation can vary; therefore, the development of standardized protocols is necessary. This will enable researchers to effectively generate and apply stem cell derived HLCs on a large scale for basic and clinical research.

A hepatic progenitor differentiation system was developed that can be applied to both human embryonic and induced pluripotent stem cell lines using easy-to-follow guidelines. This procedure yields homogenous populations of hepatic progenitors in varying cultureware formats, ranging from cell culture flasks to 96 well plates. Provided below is the protocol to produce stem cell-derived hepatic progenitors in 24 and 96 well formats.

Cell density used in the protocol presented below is specified for one well of a 24 and 96 well plate respectively (see **Table 1**). Optimization of the starting cell number is required for the different cell culture plate formats and cell lines. Suggested starting cell density for protocol optimization is 2 x 10^5 cells/cm^2. For density optimization, several cell densities can be tested by adding ± 50,000 cells/cm^2 at a time.

PROTOCOL:

1. Human pluripotent stem cell (hPSC) maintenance on laminin-521

1.1 Maintain human pluripotent cells (hPSCs) at 37 °C and 5% CO_2 in a 6 well plate on laminin-521 (LN-521). Feed the cells daily with 2 mL of stem cell maintenance medium (i.e., mTeSR1 medium) per well of a 6 well plate up to the chosen seeding day for differentiation (day 0).

1.2 Ensure that the desired cell confluency of 70–80% is achieved prior to cell harvesting.

2. Laminin-521 multiwell preparation and hPSC seeding for differentiation

NOTE: For hPSCs not maintained on LN-521 (e.g., matrigel or fibronectin), split hPSCs onto LN-521 and culture for 1 week prior to passaging and eliciting differentiation to improve the efficiency of the process[15,17,18].

2.1 Laminin-coated plate preparation

2.1.1 Thaw a vial of recombinant LN-521 (100 µg/mL) at 4 °C for 2 h or overnight.

2.1.2 Prepare an 8 µg/mL solution by diluting the thawed LN-521 in ice-cold 1x DPBS with Ca^{2+}/Mg^{2+}.

2.1.3 Add 0.25 mL of the 8 µg/mL LN-521 solution to each well of a 24 well plate or 0.05 mL to each well of a 96 well plate. Rock the plate gently from side to side to evenly coat the wells with the LN-521 solution.

NOTE: For the 96 well plate format, volume dispensing, cell seeding, and medium changes can be performed using a semiautomated pipeline. For details see Meseguer-Ripolles et al.[19].

2.1.4 Seal the LN-521 coated plates with a semitransparent, flexible film and store at 4 °C overnight prior to use.

NOTE: LN-521 coated plates can be used for up to 2 weeks when stored at 4 °C. Avoid any drying of the laminin-coated wells.

2.2 On the day of the cell seeding, warm up the precoated plates in a cell culture incubator at 37 °C for 30–60 min.

2.3 Aspirate the LN-521 solution.

NOTE: Avoid direct contact of the aspirator with the bottom of the well to prevent damage to the LN-521 coating.

2.4 Dispense 0.5 mL of stem cell maintenance medium with freshly supplemented 10 µM Rho-associated kinase (ROCK) inhibitor Y27632 to each well of a 24 well plate or 0.05 mL to each well of a 96 well plate. Place the plate in the incubator until ready for cell seeding.

2.5 On the scheduled seeding day (day 0) and with an hPSC confluency between 70–80%, mark any regions of spontaneous differentiation on the bottom of the wells of the 6 well plate.

NOTE: Spontaneous differentiation can be visualized by a gross change in the cell size and/or the presence of different cell morphologies using phase contrast microscopy.

2.6 Aspirate and discard the marked regions of differentiation and the spent medium from the wells. Wash each well with 1 mL of DPBS without Ca^{2+}/Mg^{2+} at room temperature (RT).

2.7 Add 1 mL of enzyme free dissociation reagent (see **Table of Materials**) to each well and incubate at 37 °C for 8–10 min until cells visibly detach from the plate.

2.8 Use a cell scraper to gently detach the cells from the wells. Pipette the contents of each well up and down 2–4x with a P1000 pipette to yield a single-cell suspension. For each cell line, pool cells from all maintenance wells into a sterile 50 mL tube.

2.9 Wash each emptied well with 1 mL of the stem cell maintenance medium. Add the washes to the corresponding tube containing the pooled cells from the appropriate cell line.

2.10 For each cell line, perform three viable cell counts on the pooled samples. Calculate the average live cell count (live cells/mL) for each cell line.

2.11 Centrifuge the pooled samples at 250 x *g* for 5 min at RT. Aspirate the supernatant, then resuspend the cell pellet in 1–3 mL of RT stem cell maintenance medium, freshly supplemented with 10 µM ROCK inhibitor Y27632.

2.12 For each cell line, calculate the cell number needed per the number of wells prepared at step 2.4 (see **Table 1**). Resuspend the required cell number with stem cell maintenance medium freshly supplemented with 10 µM ROCK inhibitor Y27632.

2.13 Add the calculated volume(s) into the wells of the prepared and precoated plates from step 2.4 without removing the volume added previously. The total volume per well will be 1 mL for a 24 well plate and 0.1 mL for a 96 well plate. Gently rock the plates from side to side and back and forth to ensure even cell dispersion throughout the well.

NOTE: An even cell distribution across the well is key to ensure homogenous cell seeding and successful differentiation.

2.14 Place the seeded plates into the incubator and immediately rock the seeded plates gently back and forth and from side to side to evenly distribute the cells and maintain cultures at 37 °C and 5% CO_2.

3. Differentiating hPSCs to hepatic progenitors on laminin-521

3.1 Prepare media for definitive endoderm induction (stage 1) using the Endoderm Basal Medium supplemented with the correct additives.

3.1.1 On day 0, thaw the bottle of the Endoderm Basal Medium overnight at 4 °C.

3.1.2 Prepare Stage 1 Medium 1 (for use on day 1) as needed.

3.1.3 Thaw Supplement MR and Supplement CJ on ice.

3.1.4 Dilute Supplement MR and Supplement CJ 1:100 in the Endoderm Basal Medium.

3.1.5 Prepare Stage 1 Medium 2 for use on days 2–4 as needed.

3.1.6 Dilute Supplement CJ 1:100 in the Endoderm Basal Medium.

3.2 Prepare media for the subsequent hepatic progenitor cell specification (Stage 2) differentiation using the Hepatic Progenitor Medium.

3.2.1 On day 4, thaw the bottle of the Hepatic Progenitor Medium overnight at 4 °C.

NOTE: 1% penicillin/streptomycin (final concentrations of 100 IU/mL and 100 µg/mL, respectively) was used for this experiment. Antibiotics are not required; antibiotic use is at user discretion.

3.3 On day 1 of the differentiation, remove the spent stem cell maintenance medium with 10 µM ROCKi Y-27632 medium from the wells and replace with 0.5 mL of complete Stage 1 Medium 1 per well of a 24 well plate and 0.1 mL per well of a 96 well plate.

3.4 On days 2, 3, and 4, remove the spent medium and feed each well with 0.5 mL of Stage 1 Medium 2 per well for a 24 well plate or 0.1 mL per well of a 96 well plate.

3.5 On day 5, fix the wells for definitive endoderm differentiation analysis via immunocytochemistry. For the remaining wells, remove the spent medium and feed each well with 0.5 mL of Hepatic Progenitor Differentiation Medium per well for a 24 well plate or 0.1 mL per well of a 96 well plate. Refresh the medium again on days 6, 7, and 9.

3.6 On day 10, harvest wells for hepatic progenitor differentiation analysis or proceed with further hepatocyte-like cell differentiation.

NOTE: At this time point, samples were either fixed with 4% paraformaldehyde (PFA) for immunocytochemistry analysis or supernatant was collected for ELISA and cells were collected for protein quantification.

4. Characterization of the hepatic progenitor differentiation cultures generated from hPSCs on laminin-521

4.1 On day 5, detect expression of definitive endoderm-specific markers using immunostaining.

4.2 On day 10, detect expression of hepatic progenitor-specific markers using immunostaining.

4.3 On day 10, measure AFP and ALB secretion via ELISA using a kit following manufacturer's instructions and normalize per mg protein as determined by a bicinchoninic acid (BCA) protein assay.

4.4 Assess the hepatic progenitor variability of the 96 well plate by quantifying the percentage HNF4α positive cells per well.

5. Immunocytochemistry and image acquisition

5.1 On days 5 and 10 of the differentiation, wash cells with 1x DPBS 3x, with 0.5 mL per well of a 24 well plate and 0.1 mL per well of a 96 well plate. Incubate the plate with gentle shaking for 2–5 min at RT.

NOTE: Use DPSB without Ca^{2+}/Mg^{2+} for immunocytochemistry.

5.2 Fix the cells with 4% paraformaldehyde (PFA) at RT for 15–30 min by adding 0.3 mL of PFA per well for a 24 well plate and 0.1 mL per well of a 96 well plate.

5.3 Wash with 1x DPBS 3x as described in step 5.1.

5.4 Permeabilize the membrane with PBST using 0.1% Tween, 1x DPBS and incubate for 20 min at RT by adding 0.3 mL of PBST per well of a 24 well plate and 0.1 mL per well of a 96 well plate.

5.5 Perform the protein block by incubating the cells with 10% BSA in PBST for 1 h, adding 0.3 mL of BSA per well of a 24 well plate and 0.1 mL of BSA per well of a 96 well plate, gently shaking using a plate shaker.

5.6 After protein blocking, replace the blocking solution with the primary antibody diluted in 1% BSA in PBST and incubate at 4 °C with gentle shaking overnight.

NOTE: Do not wash between protein block and antibody addition.

5.7 After 24 h, wash wells 3x with PBST.

5.8 Add the secondary antibody in 1% BSA in PBST. Incubate 1 h at RT in the dark with gentle shaking.

5.9 After secondary antibody incubation, wash wells 3x with 1x DPBS and dispense Hoechst stain according to the manufacturer's instructions for 10 min at RT in the dark with gentle shaking.

5.10 Wash 3x with DPBS. The plates are now ready for imaging.

NOTE: Store plates at 4 °C in the dark until imaging.

5.11 Image the multiwell plate using a high-content imaging microscope after immunohistochemistry. Image acquisition of several fields of view is recommended to obtain a true representation of the well. The expression of the different markers was assessed via cell segmentation analysis using commercial software (see **Table of Materials**) (**Figure 1**).

NOTE: Cell segmentation can also be performed using an image analysis open-source software such as CellProfiler or Fiji[20,21].

REPRESENTATIVE RESULTS:

Hepatic progenitor differentiation from both hESC (H9) and hiPSC (P106) lines was performed following the stepwise protocol described in **Figure 2**. Here, pluripotent stem cells were seeded as single cells into LN-521-coated plates prior to the start of the differentiation. Cell confluency is the key for a robust and reproducible differentiation. Once the right confluency was achieved (**Figure 2**), differentiation was initiated. At day 5, definitive endoderm specification was assessed via Sox17 expression. In both cell lines, Sox17 was highly expressed with 80% ± 0.5% and 87.8% ± 0.5% SEM of Sox17-positive cells for H9 and P106, respectively (**Figure 3**). At day 10, hepatic progenitors displayed a cobblestone-like morphology (**Figure 2**). In addition, hepatic progenitor specification was assessed for HNF4α, AFP, ALB, and cytokeratin-19 (CK19) expression as well as AFP and ALB protein secretion[10,15,22] (**Figure 4**). Both H9 and P106 hepatic progenitor cultures expressed fetal hepatic markers such as HNF4α (91% ± 0.5% and 90% ± 0.2%), AFP (89.7% ± 1.8% and 86% ± 1.2%), and CK19 (78.5% ± 3.2% and 83.6 ± 1.8%) (**Figure 4**). AFP secretion was detected at day 10 in both cell lines (32.4 ± 1.6 and 47.8 ± 5.9 ng/mL/mg/24 h) (**Figure 5**). Albumin synthesis was observed at lower levels (30.7% ± 1.8% and 27.2% ± 1.1%) (**Figure 4**) and was not detected via ELISA (**Figure 5**).

The protocol allowed the standardized production of hepatic progenitors from 24 well to 96 well plates. A semiautomated pipeline was employed to produce 96 well plates of hepatic progenitors from H9 and P106 cell lines as previously described[17]. Cell number variability and hepatic progenitor differentiation efficiency was assessed via quantification of HNF4α expression. At day 10, hepatic progenitors showed no significant variability across rows with >94% of HNF4α-positive cells per well for H9 and 97% HNF4α-positive cells for P106 (**Figure 6**).

FIGURE LEGENDS:

Figure 1: Cell segmentation pipeline overview. (**A**) Using the original image, (**B**) nuclear staining was used for nuclei segmentation. (**C**) A nuclear segmentation quality control step based on shape and size was performed to only quantify clearly segmented nuclei. (**D**) Following this, positive HNF4α stained nuclei were quantified. (**E**) Finally, an intensity-based threshold was employed to identify HNF4α-expressing cells. In **C** and **E**, green nuclei represent

selected cells and magenta nuclei indicate discarded cells. Scale bar = 50 µm.

Figure 2: Hepatic progenitor differentiation from hPSCs. (**A**) Schematic representation of the hepatic progenitor differentiation protocol. (**B**) Representative images highlighting the morphological changes during the differentiation. At day 0 (D0), hPSCs presented a packed monolayer of cells. Following this, hPSCs were primed into definitive endoderm on day 5 (D5). This was followed by hepatic progenitor differentiation on day 10 (D10). Hepatic progenitors displayed a cobblestone-like cell morphology**.** Scale bar = 75 µm.

Figure 3: Characterization of definitive endoderm specification. At day 5, cells were stained for Sox17, a definitive endoderm marker. The percentage of Sox17-positive cells was 80 ± 0.5% for H9 and 87.8 ± 0.5% for P106. Percentage quantification was based on 10 separate wells with 6 fields of view per well. Data are shown as the average ± SEM. Scale bar = 50 µm.

Figure 4: Hepatic progenitor characterization. At day 10, hepatic progenitors were stained for hepatic markers (**A**) HNF4α, (**B**) AFP, and (**C**) ALB. For H9, the percentage of positive cells were 91% ± 0.4%, 89.7% ± 1.8%, and 30.7% ± 1.8% for HNF4α, AFP, and ALB, respectively. For P106, the percentage of positive cells were 90% ± 0.2%, 86% +/- 1.2%, and 27.2% ± 1.1% for HNF4α, AFP, and ALB, respectively. (**D**) Cholangiocyte lineage potential was assessed via CK19 expression; H9-derived hepatic progenitors expressed 78.5% ± 3.2% CK19-positive cells, whereas 83.6% ± 1.8% of CK19-positive cells were observed for P106 hepatic progenitors. Immunoglobulin G (IgG) staining was used as a staining control. Percentage quantification was based on 10 separate wells with 6 fields of view per well. Data are shown as the average ± SEM. Scale bar = 50 µm.

Figure 5: Hepatic progenitor protein secretion analysis. The secretion of alpha fetoprotein (AFP) and albumin (ALB) was analyzed in hepatic progenitor cultures at day 10 in H9 and P109. The data represent three biological replicates and the error bars represent the SD. Secreted proteins were quantified from 24 h culture medium as nanograms of secreted protein per mL per mg of protein, n = 3; ND = not detected.

Figure 6: Assessment of well-to well variability in 96 well plate. (**A**) Visualization of a 96 well plate view of H9-derived hepatic progenitors stained with HNF4α. (**B**) Quantification of the HNF4α-positive cells. Average of cell number per well in rows, from six fields of view per well quantified. The average cell number across the plate was 94.81% ± 0.22 SEM HNF4α-positive cells per well. No statistically significant differences were observed between wells. (**C**) Visualization of a 96 well plate view of P106-derived hepatic progenitors stained with HNF4α. (**D**) Quantification of HNF4α-positive cells. The average cell number per wells in rows, from six fields of view per well and quantified. The average cell number across the plate was 97.7% ± 0.57 SEM HNF4α-positive cells per well. No statistically significant differences were observed between rows. Well H12 was used as an Immunoglobulin G (IgG) staining control. Scale bar = 1 mm. One-way ANOVA with Tukey's post-hoc statistical tests were employed.

Table 1: Recommended cell density for the different plate formats for the hPSC cell lines

used in this protocol.

DISCUSSION:

The generation of human hepatic progenitor cells from pluripotent stem cells on a large scale could represent a promising alternative to cadaver-derived material. Protocol standardization and reproducibility are key to ensure technology translation and impact for biomedical research. To address this, previous work has focused on developing a stepwise differentiation protocol from hESC and iPSCs using defined additives and matrices[15,23–28]. By doing this, hepatocyte phenotype and reproducibility have been improved, permitting the semiautomation of the differentiation process[19]. The system presented is strengthened by its combination of off-the-shelf cell culture media and a facile hepatocyte differentiation system.

Previously, pluripotent cell density prior to the start of the differentiation protocol was highlighted as a key variable to achieve a homogenous population of hepatic progenitor cells[26]. Using this more refined procedure, it is possible to generate large numbers of stem cell-derived hepatic progenitors in a stepwise manner using a range of cell densities. At day 5, definitive endoderm induction was validated by Sox17 staining (**Figure 3**). Efficient and robust differentiation into definitive endoderm was achieved with both tested ESC and iPSC lines, with more than 80% expressing Sox17 (**Figure 3**). At day 10, hepatic progenitors displayed a uniform cobblestone-like morphology, and liver stem cell markers were highly enriched for both AFP and HNF4α (>86%, **Figure 4**). Using a combination of manual and semiautomated technologies it was possible to perform differentiation in multiple plate formats[19].

In its current form, cell differentiation is suitable for in vitro based experimentation. However, cell enrichment would likely be required before clinical application to ensure that a homogenous population of hepatic progenitors are prepared for delivery.

In conclusion, the protocol described here provides the field with a standardized approach to produce hepatic progenitors on a large scale. Future work will focus on the production of a new medium for subsequent HLC differentiation, maturation, and maintenance.

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DISCLOSURES:

David C. Hay is a co-founder and shareholder of Stemnovate Ltd. The rest of the authors certify that they have no conflicts of interest in the subject matter or materials discussed in this article.

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Figure 1

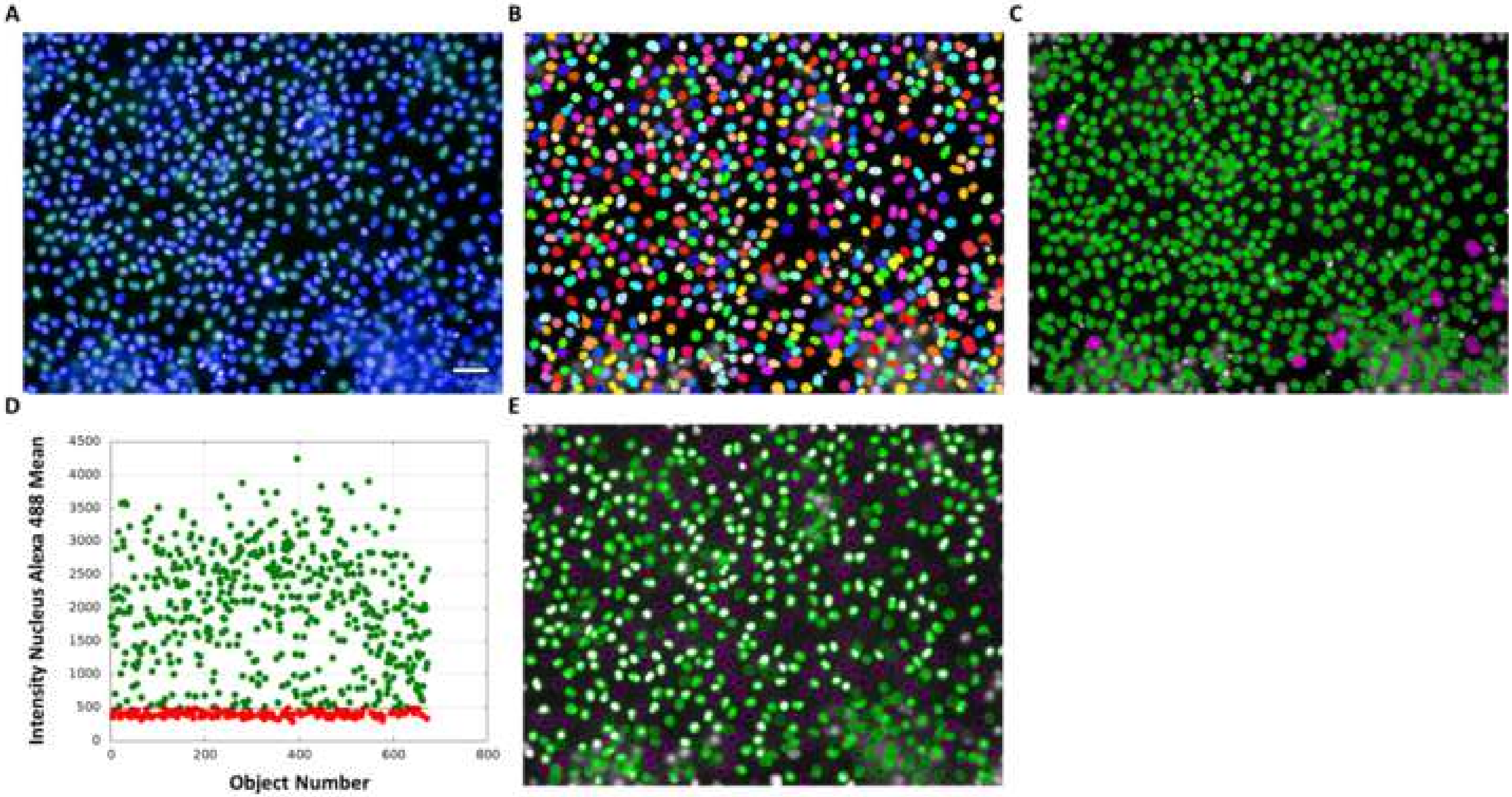
A
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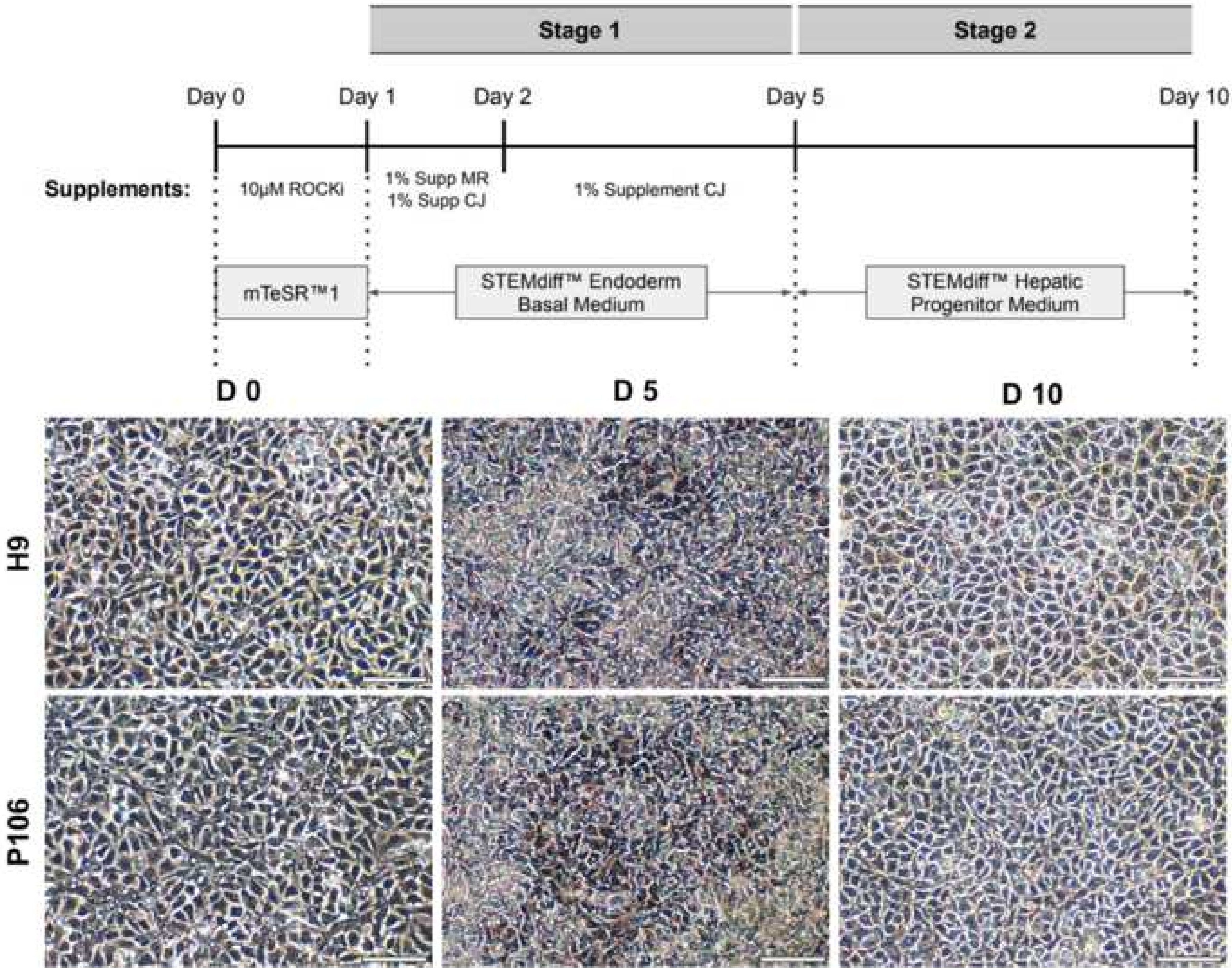
Figure 2



Stage 1
Stage 2
Day 0
Day 1
Day 2
Day 5
Day 10
Supplements:
10µM ROCKi
1% Supp MR
1% Supp CJ
1% Supplement CJ
mTeSR™1
STEMdiff™ Endoderm Basal Medium
STEMdiff™ Hepatic Progenitor Medium
D 0
D 5
D 10
H9
P106

Figure 3

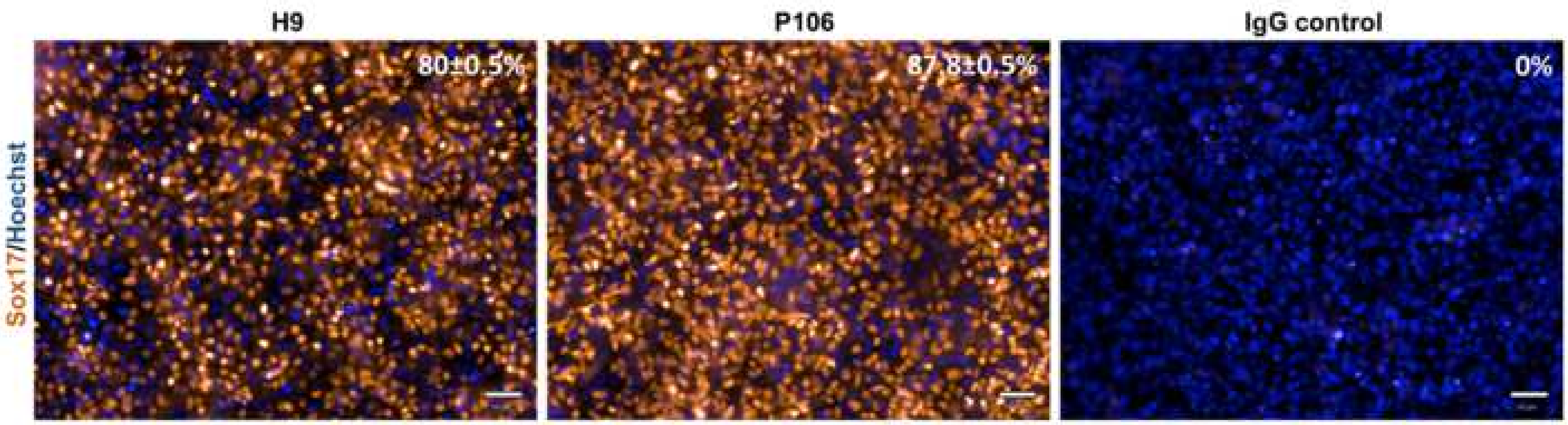
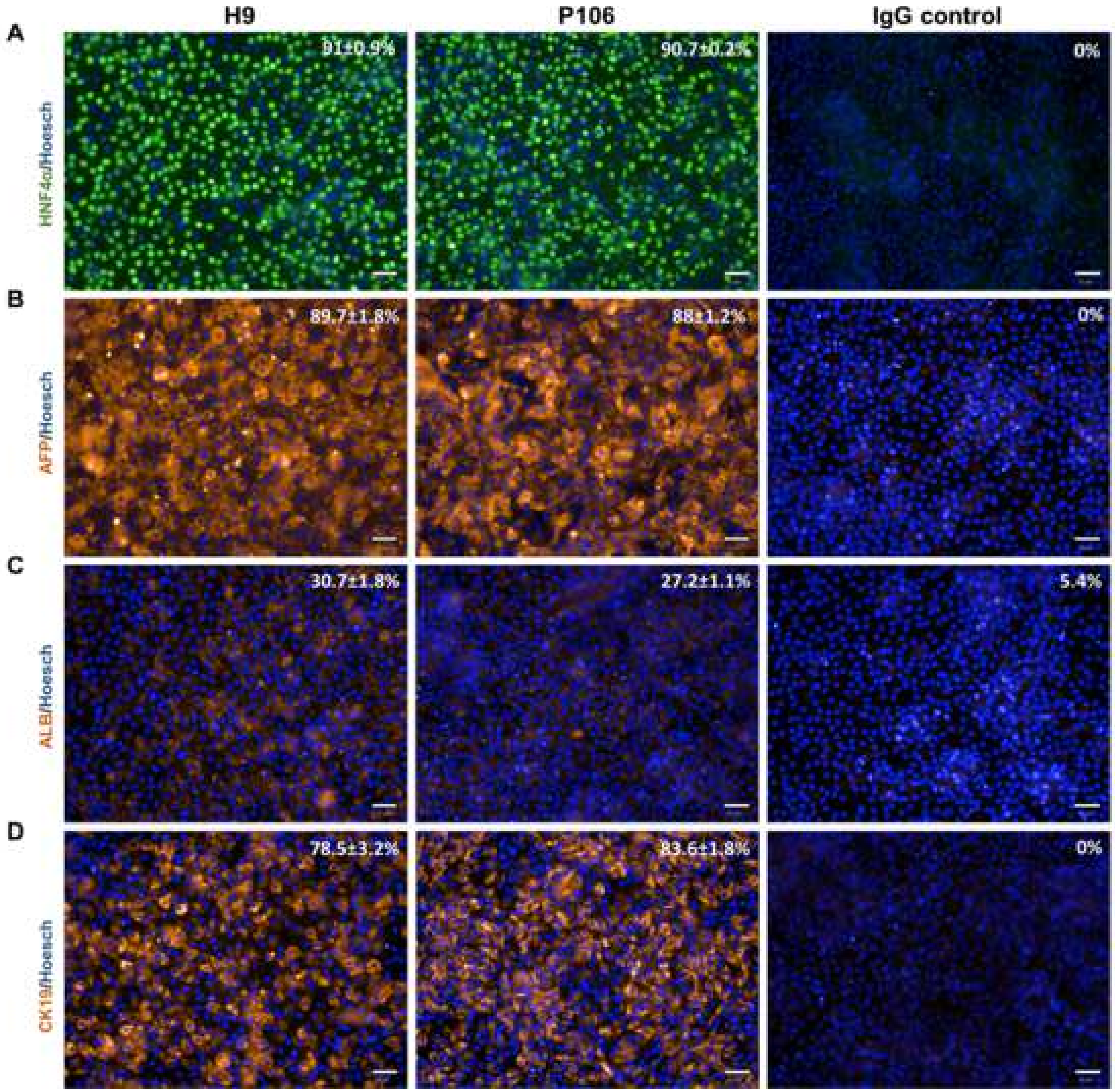
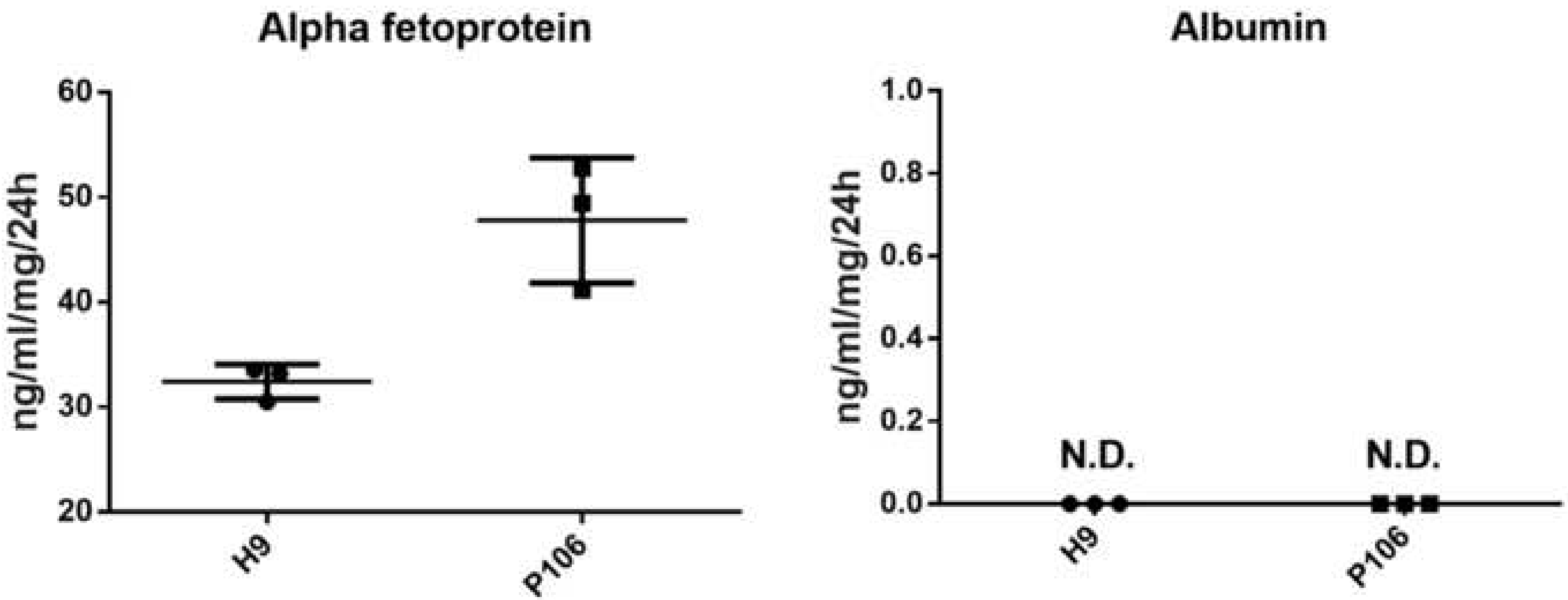


Figure 4



H9
P106
IgG control
A
B
C
D
HNF4α/Hoesch
AFP/Hoesch
ALB/Hoesch
CK19/Hoesch
91±0.9%
90.7±0.2%
0%
89.7±1.8%
88±1.2%
0%
30.7±1.8%
27.2±1.1%
5.4%
78.5±3.2%
83.6±1.8%
0%



Alpha fetoprotein
ng/ml/mg/24h
60
50
40
30
20
H9
P106
Albumin
ng/ml/mg/24h
1.0
0.8
0.6
0.4
0.2
0.0
N.D.
N.D.
H9
P106

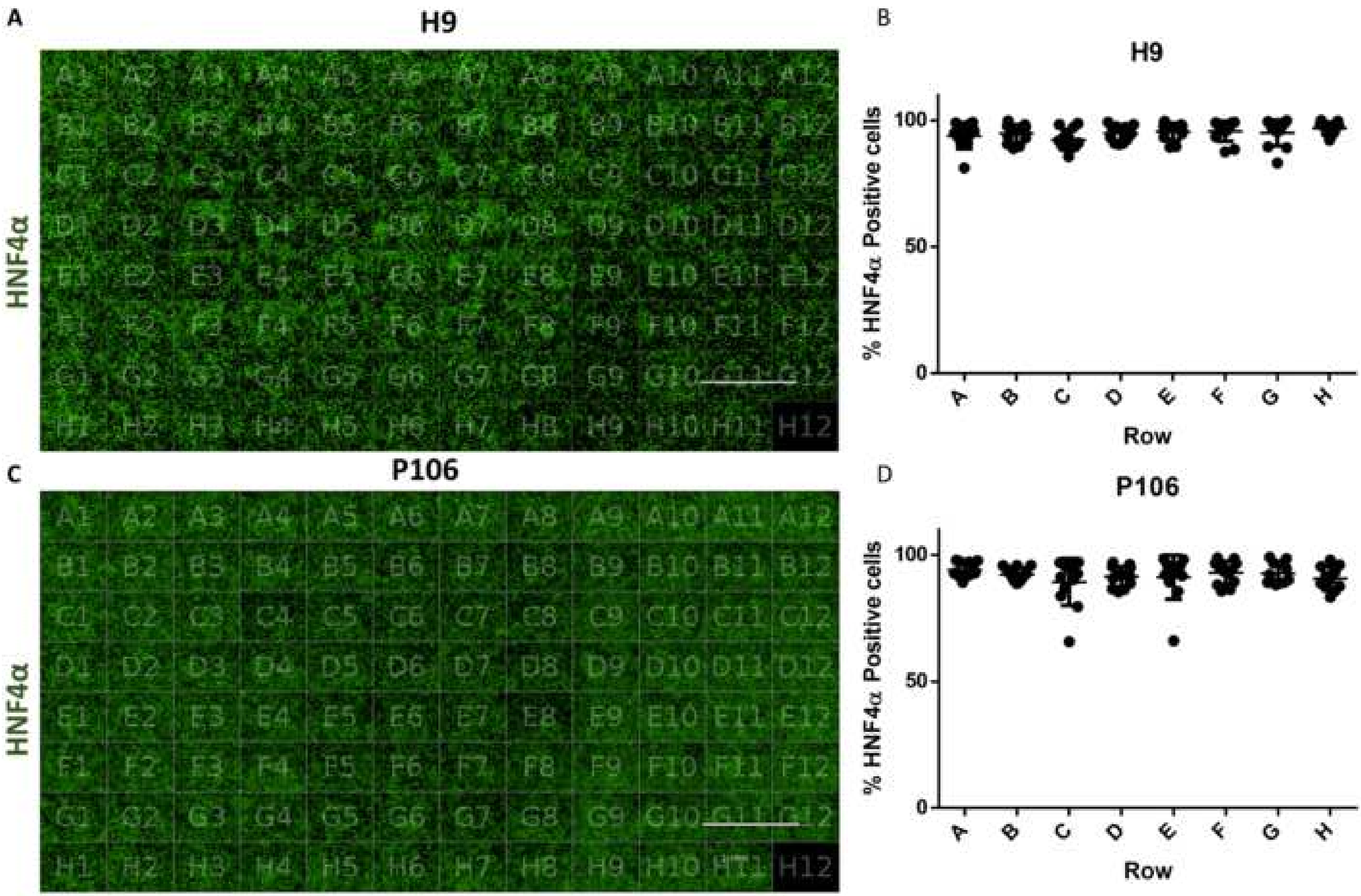

A
H9
HNF4α
B
H9
% HNF4α Positive cells
100
50
0
A
B
C
D
E
F
G
H
Row
C
P106
HNF4α
D
P106
% HNF4α Positive cells
100
50
0
A
B
C
D
E
F
G
H
Row

Plate format	Surface area (cm2)	Cells per cm2	Total cells per well	Dispensing volume (mL)
24-well plate	1.9	210526	400000	0.5
96-well plate	0.32	187500	60000	0.05

Cell concentration (cells/ml)
800000
1200000

Name of Material/ Equipment	Company	Catalog Number	Comment
DPBS with Calcium and Magnesium	ThermoFisher	14040133	
Gentle cell dissociation reagent	STEMCELL Technologies	7174	
Hoechst 33342 Ready Flow Reagent	thermofisher	R37165	
Human Recombinant Laminin 521	BioLamina	LN521-02	
Human Serum Albumin ELISA	Alpha Diagnostics	1190	
Human Serum Alpha Fetoprotein ELISA	Alpha Diagnostics	500	
mTeSR1 medium	STEMCELL Technologies	5850	
Operetta High-Content Imaging System	PerkinElmer	HH12000000	
PBS, no calcium, no magnesium	ThermoFisher	14190250	
Penicillin-Streptomycin (10,000 U/mL)	Life Technologies	15140122	
Rho-associated kinase (ROCK)inhibitor Y27632	Sigma-Aldrich	Y0503-1MG	
STEMdiff Definitive Endoderm Supplement CJ	STEMCELL Technologies		
STEMdiff Definitive Endoderm Supplement MR	STEMCELL Technologies		
STEMdiff Endoderm Basal Medium	STEMCELL Technologies		
STEMdiff Hepatic Progenitor Medium	STEMCELL Technologies		
TWEEN 20	Sigma-Aldrich	P9416	
Antibodies			
Albumin	Sigma-Aldrich	A6684	1:200 (mouse)
Alpha-fetoprotein	Sigma-Aldrich	A8452	1:400 (mouse)
HNF-4α	Santa Cruz	sc-8987	1:400 (rabbit)
IgG	DAKO		1:400
Sox17	R&D Systems, Inc.	AF1924	1:200 (Goat)
Software			
Columbus Image Data Storage and Analysis system	PerkinElmer		

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Dear Dr Bajaj,

We are grateful for the editorial comments for the manuscript "Hepatic progenitor specification from pluripotent stem cells using a robust differentiation system", for publication in JoVE. We have fully addressed the editorial comments, which we believe has improved the manuscript. Please let me know if you require any further information, and I look forward to hearing back from you in due course.

Kind regards,

Dr Jose Meseguer Ripollés

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

The new formatting has been implemented for addressing the editorial comments.

2. Please address all the minor specific comments marked in the manuscript.

All minor comments have been revised.

3. Please check with your funding source regarding PMC deposition. We do not deposit articles into PubMed Central on behalf of the authors. However, authors can self-deposit into PMC if required by their funding source.

We are grateful for the PMC deposition notice; this will be performed by the specific funding sources.