Stem Cell Research

SUPPLEMENTAL INFORMATION

Non-Colony Type Monolayer Culture of Human Embryonic Stem Cells

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SUPPLEMENTAL MATERIALS AND METHODS

Cell Culture Medium and Other Reagents

The reagents used in this study include cell culture medium DMEM/F12, Knockout Serum Replacer (KSR), DMEM medium, and Dulbecco's Phosphate-Buffered Saline (D-PBS) free of Ca^{2+}/Mg^{2+} from Invitrogen Inc. (Calsbad, CA); mTeSR™1 and TeSR™2 (animal protein-free) basal medium and their supplements from StemCell Technologies (Vancouver, BC, Canada); heat-inactivated fetal bovine serum (FBS) from Gemini (Gemini Bio-Products) and Hyclone (Logan, Utah); JAK inhibitor I (JAKi, Product No., 420099), SB 203580 (p38MAPK inhibitor or p38i, Product No., 559389), and Y-27632 (ROCK inhibitor or ROCKi, Product No., 688000) from Calbiochem (San Diego, CA).

Antibodies

Antibodies against cytokeratin 8 [CK8 (C51), mouse IgG1, sc-8020], α-fetoprotein (AFP, mouse IgG2a, sc-8399), FOXA2 [HNF-3β (P-19), goat polyclonal antibody, sc-9187), Oct-4 (mouse IgG2b, sc-5279), SSEA-1 (mouse IgM, sc-21702), and SSEA-4 (mouse IgG3, sc-21704) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); albumin (rabbit polyclonal antibody, Cat No. A0001) and smooth muscle actin (clone 1A4, mouse IgG2a) from DakoCytomation Inc. (Capinteria, CA); troponin T (Av-1, mouse IgG1, Ref: MS-295-P0) and desmin (rabbit IgG, Ref RB-9014-P1) from Thermo Scientific; albumin (clone HSA1/25.1.3, mouse IgG1, product code CL2513A) from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada); GFAP (13-0300) from Zymed (San Francisco, CA); HNF4α (C11F12, rabbit monoclonal antibody) from Cell Signaling Technologies, Inc. (Danvers, MA); MAP2 (Cat No. AB5622) from Millipore (Billerica, MA); NANOG from COSMO Bio Co. Ltd. (Japan); nestin (MAB5326, rabbit polyclonal antibody) from Chemicon International (Temecula, CA); and TUBB3 (Tuj1, mouse IgG2a, MMS-435P) from Convance Inc. (Princeton, New Jersey). Both Tra-1-60 and Tra-1-81 were kind gifts of Dr. Peter Andrews (Sheffield University, United Kingdom).

Human ES Cell Culture on Mouse Embryonic Fibroblasts (MEFs)

MEFs (CF1 strain), passage number 5 and 6 (designated as p5 and p6), were cultured in DMEM medium (supplemented with 10% FBS, 2 mM L-glutamine, and 0.1 mM non-essential amino acids) and irradiated at a dose of 8,110 rads with an X-ray machine (Faxitron X-ray Corporation, Wheeling, IL). The irradiated cells were plated at a density of 1.88×10^5 cells per well (i.e., 1.96×10^4 cells/cm^2) on a 6-well polystyrene plate (Cat No. 353046, Becton Dickinson Labware, Franklin Lakes, NJ) coated with 0.1% gelatin and incubated at 37°C for 24 h. Human ES cells were plated on top of MEF feeder layers as small clumps (~ 50 to 100 μm in diameter) in hESC medium containing 80% DMEM/F12 medium, 20% KSR, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, and 4 ng/ml of FGF-2 (R&D Systems, Minneapolis, MN). For cell passage, hESC colonies (at day 3 or day 4) that contained morphologically differentiated cells (~ 5%) were manually removed, and the remaining colonies were rinsed twice with D-PBS, and incubated with 1 mg/ml collagenase IV (Invitrogen) in hESC medium for 10 to 30 min, until the colonies began to detach. Colonies were removed to a 15 ml tube with extra hESC medium and allowed to sediment at room temperature for 5 to 10 min. The supernatants containing residual MEFs were removed and the colonies resuspended with 5 ml hESC medium to repeat the sediment step. Finally, the colonies were triturated into small clumps and plated on the 6-well plate.

Feeder-free Culture of HESCs

MEF-conditioned medium (MEF-CM) was generated by plating X-ray-irradiated MEFs (at p5) onto 0.1% gelatin-coated flasks at a density of 1×10^7 cells per 175-cm^2 culture flask. At next day, MEFs were rinsed with D-PBS and replenished with 25 ml of hESC medium containing 4 ng/ml of FGF-2. The supernatants were collected from day 2 to day 7, mixed, filtered, and stored in –20°C freezer within two months. The 6-well plates were coated with 2.5 to 7.5% BD Matrigel (BD Biosciences, Bedford, MA) in DMEM/F12. After overnight incubation at 4°C, the Matrigel medium was removed and hESC clumps were seeded either in the presence of MEF-CM (supplemented with an additional 4 ng/ml of FGF-2) or in normal hESC medium containing 100 ng/ml of FGF-2. The medium was changed daily for 3 to 4 days. For passage, the cells were rinsed with D-PBS and treated with 2 mg/ml dispase (Invitrogen Inc.) at 37°C for 15 min. Small hESC clumps were generated by trituration and plated on the Matrigel for desired experiments.

Non-colony Type Monolayer (NCM)-Based Xeno-Free Culture of HESCs

When adapting various lines to the NCM-based xeno-free system, we found that the amine type of BD PureCoat Carboxyl plasticware (BD Biosciences) coated with fibronectin worked better than the carboxyl. We initially used 50% MEF-CM/mTeSR™1 medium for the NCM culture. Subsequently, we were able to substitute hESC medium containing Xeno-free Knockout Serum Replacer (X-KSR, Invitrogen Inc.) for the MEF-CM and TeSR2 for mTeSR™1, resulting in a defined xeno- and feeder-free protocol. Medium was further optimized to 10% X-KSR in TeSR2 and finally to 2% Xeno-free Growth Factor Cocktail Supplement (XGFS; Invitrogen) in TeSR2. Additionally, using a 96-well format, we tested the effects of different sources and concentrations of fibronectin on plating efficiency.

High-Density Single-cell Plating Efficiency (SCPE) Assays

Dissociated hESCs were seeded at high density (e.g., 2×10^5 to 2×10^6 cells per well) in 6-well plate) in the presence or absence of small-molecules. At 24 h, the medium was removed and plated cells were dissociated with Accutase™. The cells were then stained with 0.2% Trypan Blue Stain (Invitrogen) and counted under microscope with a hemocytometer or counted by Countess® Cell Counter (Invitrogen).

Array-based Comparative Genomic Hybridization (aCGH)

Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Human Genome CGH Microarray Kit 244A was obtained from Agilent Technologies, Inc. (Santa Clara, CA). Approximately 3 μg genomic DNA was used for hybridization. The CGH was performed using the software also developed by Agilent Technologies. Other reagents, except for control human DNA (Promega), and procedures were essentially as described previously (Tesar et al., 2007).

RNA Preparations

RNA was extracted by using the basic Trizol protocol (Invitrogen Inc.) with slight modifications. Briefly, hESC cells were dissolved in 1 ml of the Trizol solution and stored in -80°C freezer prior to use. Human ES cell lysates were further subjected to chloroform extraction and

isopropanol precipitations. A DNA-Free kit (Applied Biosystems/Ambion, Austin, TX) was used to remove genomic DNA contamination. The quality of RNAs was agarose gel-verified and concentration determined by using the ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

Complementary DNA (cDNA) Microarray

The reagents and softwares for cDNA microarray were purchased from Agilent Technologies (Santa Clara, CA). Genome-wide mRNA expression was analyzed as described previously (Tesar et al., 2007).

Flow Cytometry

Single-cell suspensions were prepared from hESC colonies on MEFs and cells from the NCM culture. The cell pellets were rinsed once with D-PBS, resuspended in FACS buffer (DMEM/F12 medium containing 10% FBS), and incubated on ice for surface marker staining. The cells used for localization of intracellular markers were fixed in 4% paraformaldehyde, incubated at room temperature for 10 min, and then resuspended in 0.1% Triton X-100 followed by incubation on ice for 15 min and centrifugation. The pellets were then resuspended in FACS buffer and incubated with desired primary antibodies in a round-bottom 96-well plate (Fisher Scientific). Approximately 1 to 2.5×10^5 cells (in 50 μl of volume) were used for each reaction. Cells were incubated at 4^oC for 1 h, washed with FACS buffer, and incubated with Alexa Fluor® 488-conjugated secondary antibodies (1:100 dilutions, Invitrogen Inc.) at 4^oC for 30 min. Cells were washed, resuspended in FACS buffer, and analyzed by the FACSCalibur system with a 96-well plate HTS attachment (BD Biosciences, San Jose, CA). Data were collected and analyzed using PlateManager and CellQuest Pro software (BD Biosciences).

Neurectoderm and Neural Differentiation

The procedures for neurectoderm and neural differentiation were essentially described by Mallon and coworkers (Kozhich et al., 2012). Briefly, embryoid bodies (EBs) were derived from dissociated hESCs under NCM conditions in AggreWell™400 (StemCell Technologies) in the presence of 10 μM ROCKi. Each EB contains approximately 1000 cells. These EBs were used for neural differentiation in a neural precursor medium (NPM) supplemented with noggin and

FGF-2 and then in an FGF-2-containing medium to further propagate neural precursor cells (NPCs) (Cohen et al., 2007). Neurons were spontaneously generated by growing NPCs in the NPM without the growth factors (Kozhich et al., 2012). Neurectoderm and neural differentiation were evaluated by immunostaining the differentiated cells with specific antibodies against nestin, GFAP, and Tuj1.

Endoderm and Hepatocyte Differentiation

WA14 cells under NCM conditions were differentiated into endoderm and the hepatic lineage using a modified method based on the protocol published by Basma et al (Basma et al., 2009). Briefly, two confluent wells of the cells in 6-well plate were digested with Accutase™. These cells were then incubated overnight in a 24-well ultra-low attachment culture dish (Corning) in hESC medium containing 10 μM ROCKi. This resulted in the formation of small EBs, which were collected and replated in a 6-well low-attachment culture dish in hESC medium. After 24 h, the EBs were replated on 5% growth factor reduced (GFR) Matrigel (BD Biosciences) in DMEM/F12 medium containing 100 ng/ml activin A and 100 ng/ml FGF-2 (R&D Systems). The next day, the medium was exchanged for DMEM/F12 supplemented with 0.2% KSR and the growth factors described as above. After 48 h, the medium was replaced with DMEM/F12 supplemented with 2% KSR and the same growth factors. On the following day, the medium was switched to DMEM/F12 with 10% KSR, 0.1 mM non-essential amino acids, 1 mM L-glutamine, and 1% DMSO in the presence or absence of 100 ng/ml hepatocyte growth factor (HGF) (R&D Systems). The medium was replaced on alternate days for 8 days, at which point the cells were passed onto 5% GFR Matrigel in hepatocyte culture medium (HCM) (Lonza) supplemented with 10 ng/ml HGF, 10 ng/ml oncostatin M (R&D Systems), and 100 nM dexamethasone (Sigma-Aldrich). The cell medium was changed every other day for 9 days. Endoderm and hepatic differentiation were evaluated by immunostaining the differentiated cells with specific antibodies against multiple hepatic markers such as FOXA2, cytokeratin 8 (CK8), α-fetoprotein (AFP), and albumin (ALB). We also used a hepatic differentiation protocol described by Si-Tayeb and coworkers for both WA01 and BC1 cells under NCM conditions (Si-Tayeb et al., 2010).

Mesoderm and Cardiomyocyte Differentiation

Human ES cells grown under NCM conditions were differentiated using a spontaneous differentiation protocol. Briefly, hESC were treated with 2 mg/ml dispase and triturated as clumps. At day 1, the EBs were formed in 75-cm^2 Ultra-Low Attachment Flasks (Corning) containing alpha-MEM (Invitrogen), 20% FBS, 0.1 mM non-essential amino acids, and 2 mM glutamine. At day 2, EBs were transferred to 100-mm petri dish. The majority of EBs were attached at day 3. Alpha-MEM medium containing 20% FBS and 2 mM glutamine was changed every two days. Beating cardiomyocytes were seen (at day 23) and video-recorded. In addition, induced pluripotent stem cells (iPSCs) (e.g., BC1 cells) grown under NCM conditions were directly differentiated in the presence of alpha-MEM medium supplemented with 20% FBS. Beating cardiomyocytes were observed at day 30 and video-recorded (Supplemental Movie 2).

Plasmid DNA Transfection by Lipofectamine™ 2000

We used a transfection method based on Lipofectamine™ 2000 (Catalog. No. 11668-027, Invitrogen) to compare the transfection efficiency of plasmid DNAs between hESCs grown under NCM and the cells cultured under colony-type culture conditions. For each transfection sample, 5 μg of pmaxGFP plasmids (amaxa Inc.) and 10 μl of Lipofectamine™ 2000 solution were separately diluted in 250 μl of Opti-MEM® I Reduced Serum Medium (Invitrogen). After 5 min, the diluted reagents were mixed and incubated to form complexes at room temperature for 20 min. The complexes were then added to each well that contains hESCs and 2 ml mTeSR™1 and incubated with the cells at 37°C in a CO^2 incubator for 24 h prior to testing for GFP expression.

Lentiviral Induction of hESCs

We tested lentiviral transduction efficiency in hESCs using lentiviral particles purchased from Thermo Scientific (Lafayette, CO). Stock viral titers for SMART-MDM2-shRNA were 4.7×10^8 transforming units per ml (TU/ml) and the SMART vector control 1.8×10^9 TU/ml. At day 1, semi-confluent hESCs under the NCM condition were dissociated by Accutase™ and seeded at a density of 6 x 10^5 cells per well in a 12-well plate in mTeSR™1 medium containing 10 μM ROCKi. After 3 h, the medium was changed. The amounts of viral particles were determined by the following formula: (MOI × CN) / VT, where MOI represents the desired multiplicity of infection (MOI), CN stands for the number of cells in the well, and VT denotes stock viral titers.

Lentiviral particles that equal to 19 TU/cell were used for transducing the cells in each well. The mTeSR™1 medium was pre-warmed with 20 μg/ml polybrene for 30 min. MDM2 and vector control shRNA stocks were added in the pre-warmed medium and gently mixed. The medium was replaced with 300 μl of pre-warmed medium containing the viral particles. After 4 h incubation, additional mTeSR™1 medium (300 μl) was supplemented. After 12 to 16 h, the cells were examined for the presence of turbo-GFP, which is a maker for shRNA expression in this SMART vector system.

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LEGENDS TO SUPPLEMENTAL FIGURES

Supplemental Figure 1. Control of hESC growth rates by the non-colony type monolayer (NCM) culture. Phase images of the NCM culture of BG01 cells (passage number 10 or mcp10) at different time points after plating on 2.5% BD Matrigel with the use of 10 μM Y-27632 (ROCKi). Phase images in the middle panel were regionally enlarged view of the images in

upper panel. Cell cycle analysis (lower panel) was performed in BG01 cells grown under NCM conditions in 6-well plates at indicated time points. Representative histograms are shown.

Supplemental Figure 2. Twenty-four hour single-cell plating efficiency (SCPE) assays in WA07 (H7) cells. WA07 cells were plated on 2.5% BD Matrigel in mTeSR™1 medium containing no drug (control), 1 μM JAK inhibitor I (JAKi), and 10 μM Y-27632 (ROCKi) as indicated. The right panel is an enlarged view of the left panel.

Supplemental Figure 3. Characterization of hESCs under NCM conditions. (**A**) Karyotyping of BG01 cells cultured on MEFs for 70 passages (i.e., p70) and then grown under NCM conditions for 10 passages (mcp10). (**B**) BG01 cells (at mcp10, n = 200 cells) were examined by FISH using the probes specific for the centromeres of both chromosomes 12 and 17 (i.e., 12 Cen and 17 Cen, respectively). (**C**) Array-based CGH analysis of gene copy number variation in BG01 cells (at mcp20). (**D**) Flow cytometric analysis of hESC marker expression in BG01 cells at mcp20 and BG01 cells grown as colonies on MEFs. The arrowheads indicate the differences between BG01 cells under NCM and BG01 colonies.

Supplemental Figure 4. Indirect immunofluorescence analysis of NANOG and Oct-4 coexpression in both WA01 (H1) and WA09 (H9) cells under the non-colony type monolayer (NCM) condition.

Supplemental Figure 5. Human ES cells under NCM conditions sustain the potential to differentiate into multiple cellular fates in vitro. (**A**) Neurectoderm and neural differentiation of UC06 cells. Differentiated cells were examined with specific antibodies against β-tubulin III (Tuj1) and GFAP. The cells were co-stained with 4',6-diamidino-2-phenylindole (DAPI). The images were merged, enlarged, and presented on right panel. (**B**) Endoderm and hepatic differentiation of WA14 cells: Expression patterns of the primary hepatic markers FOXA2 (HNF-3β, in different regions of the culture) and α-fetoprotein (AFP), the intermediate hepatic markers cytokeratin 8 (CK8), and human albumin (ALB) were determined by immunofluorescence. Co-expression of AFP with CK8 or AFP with both CK8 and ALB are shown. The blue color represents Hoechst 33342 staining.

Supplemental Figure 6. The human iPSC cell line, BC1, under NCM conditions retains the potential to differentiate into multiple cellular fates in vitro. (**A**) Neurectoderm and neural differentiation of BC1 cells: Differentiated cells were examined with specific antibodies against nestin and β-tubulin III (Tuj1). The cells were co-stained with Hoechst 33342. The images were merged. (**B**) Mesodermal differentiation of BC1 cells with alpha-MEM medium containing 20% FBS: Differentiated cardiomyocytes were photographed (phase images), video-recorded (Supplemental Movie 2), and stained with specific antibodies against desmin and the myocardial cell marker, troponin. (**C**) Endodermal and hepatic differentiation of BC1 cells: Expression patterns of the hepatic markers α-fetoprotein (AFP), human albumin, and HNF4α were determined by immunostaining the cells with specific antibodies. Abbreviations: NPC, neural precursor cells; Diff, differentiation.

Supplemental Figure 7. Applications of the NCM culture. **(A)** Comparison of BG01 cell yields under NCM with the conventional colony-type culture of hESCs on MEF feeder layers. (**B**) Recovery rate of UC06 cells from cryopreservation: UC06 cells were cultured under NCM conditions, frozen in liquid nitrogen, and thawed in the presence of 1 μM JAKi or 10 μM ROCKi.

Supplemental Figure 8. Comparison of transfection and transduction efficiencies between NCM and colony-type culture (CTC) conditions. All hESCs were grown on 2.5% BD Matrigel. (**A**) Transfection efficiency of plasmid DNA. The pmaxGFP plasmids (5 μg, amaxa Inc.) were introduced into UC06 and BG01 cells by Lipofectamine™ 2000 (Invitrogen). After 24 h, the cells were fixed in 4% paraformaldehyde and stained with Hoechst 33342. GFP expression was detected by an immunofluorescence microscope and photographed. The transfection efficiency (i.e., 13% of GFP^+ cells) under NCM conditions was increased by 4.3-fold at 24 h, compared with the efficiency (i.e., 3%) under colony-type culture conditions (upper panel). A similar result was also found in UC06 cells under the same culture conditions (lower panel). (**B**) Lentiviral induction of BG01 cells. Lentiviral particles (19 TU/cell), containing the SMART vector control or SMART-MDM2-shRNAs, were used for the transduction experiments. After overnight incubation, the cells were examined for turbo-GFP (GFP) expression. Approximately 75% to

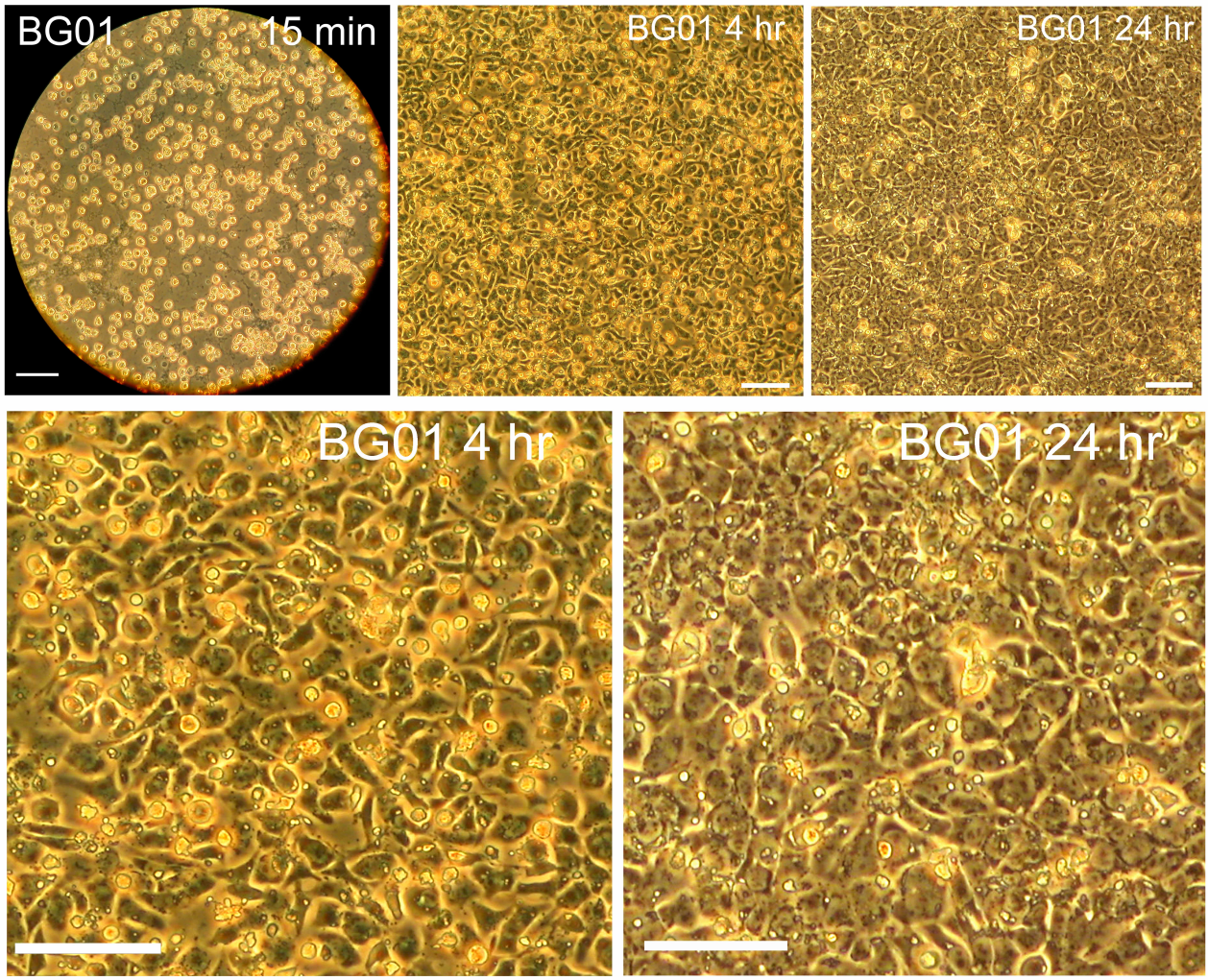
77% of BG01 cells under NCM conditions were transduced (middle and lower panels). Almost no detectable transduced signals were found in BG01 cells under colony-type culture conditions (upper panel).

LEGENDS TO SUPPLEMENTAL MOVIES

Supplemental Movie 1. Spontaneous cardiomyocyte differentiation in hESCs cultured under NCM conditions. UC06 embryoid bodies derived from NCM conditions were plated on non-coated petri dish and differentiated in alpha-MEM medium containing 20% FBS as described in Supplemental Materials and Methods. Beating cardiomyocytes were seen (at the center of the field) at day 23 and video-recorded.

Supplemental Movie 2. Spontaneous cardiomyocyte differentiation in iPSCs cultured under NCM conditions. BC1 cells under NCM were directly differentiated in alpha-MEM medium containing 20% FBS as described. Beating cardiomyocytes were recorded by time-elapse microscope and stained with specific antibodies against desmin and the myocardial cell marker, troponin (Supplemental Figure 6B).

Supplemental Figure 1



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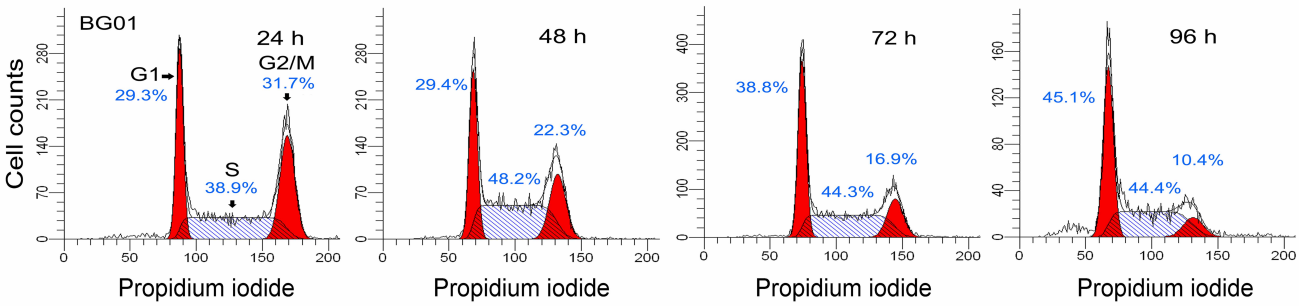
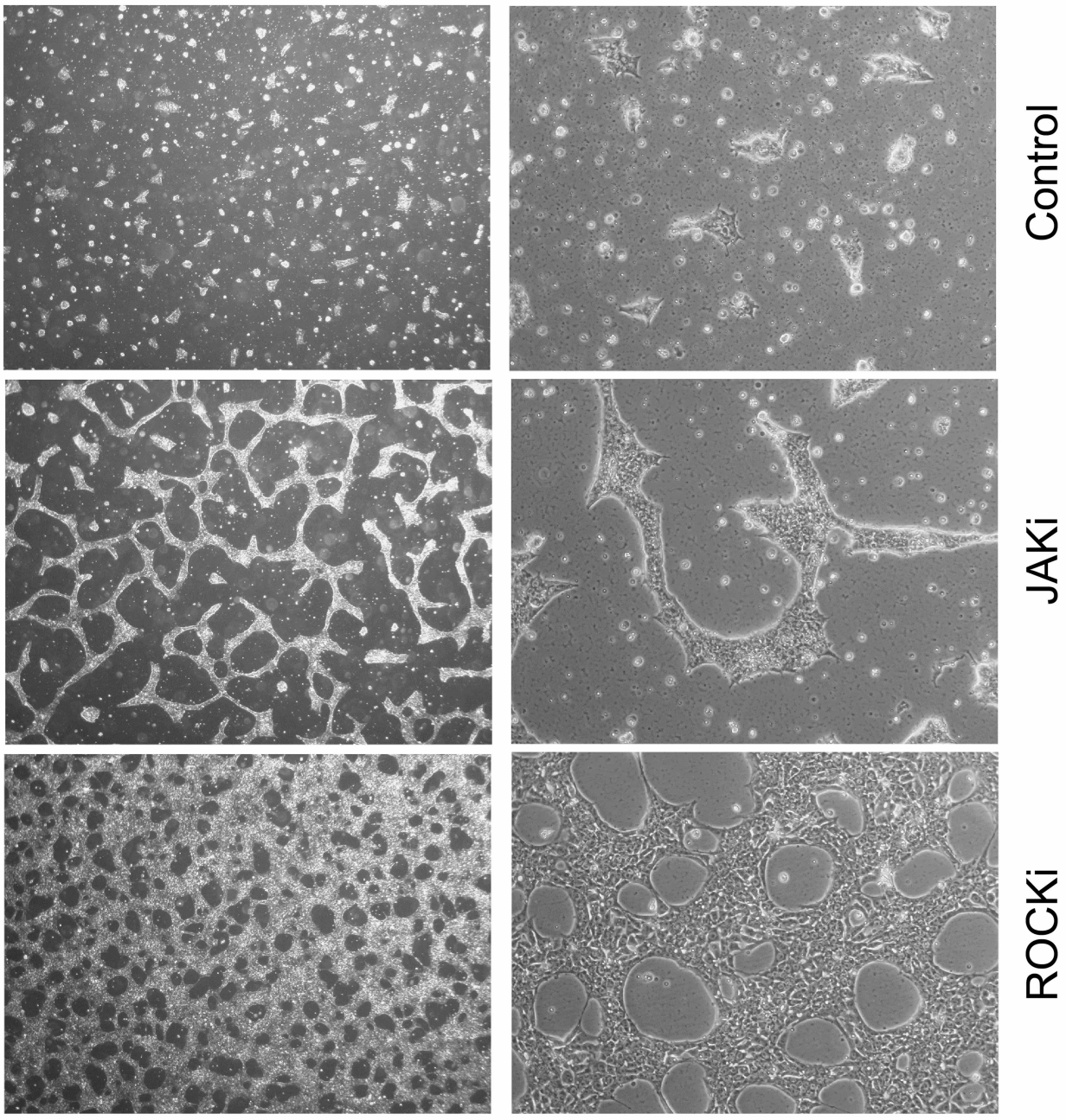
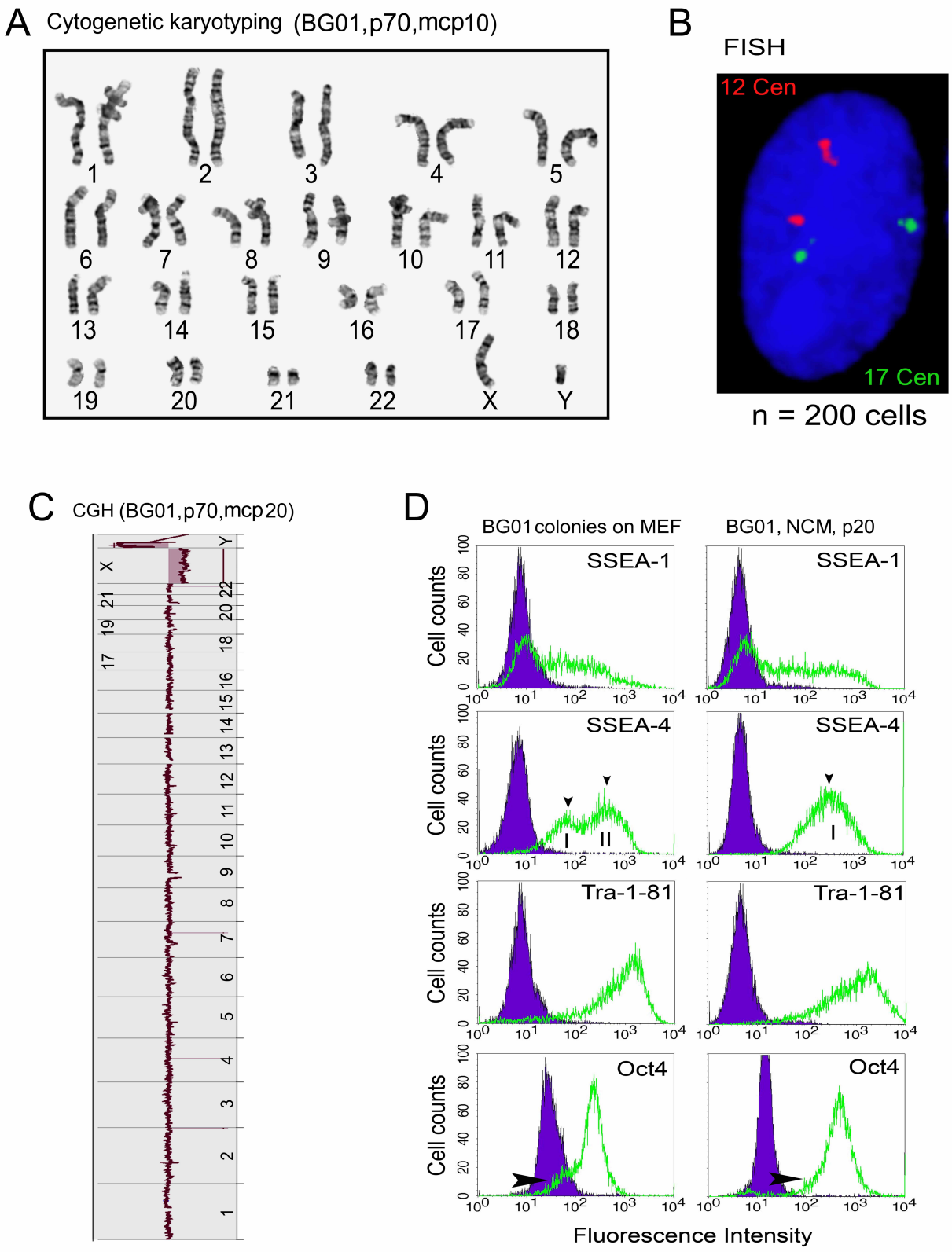


Supplemental Figure 2



24 h single-cell plating efficiency in WA07 (H7) cells

Supplemental Figure 3



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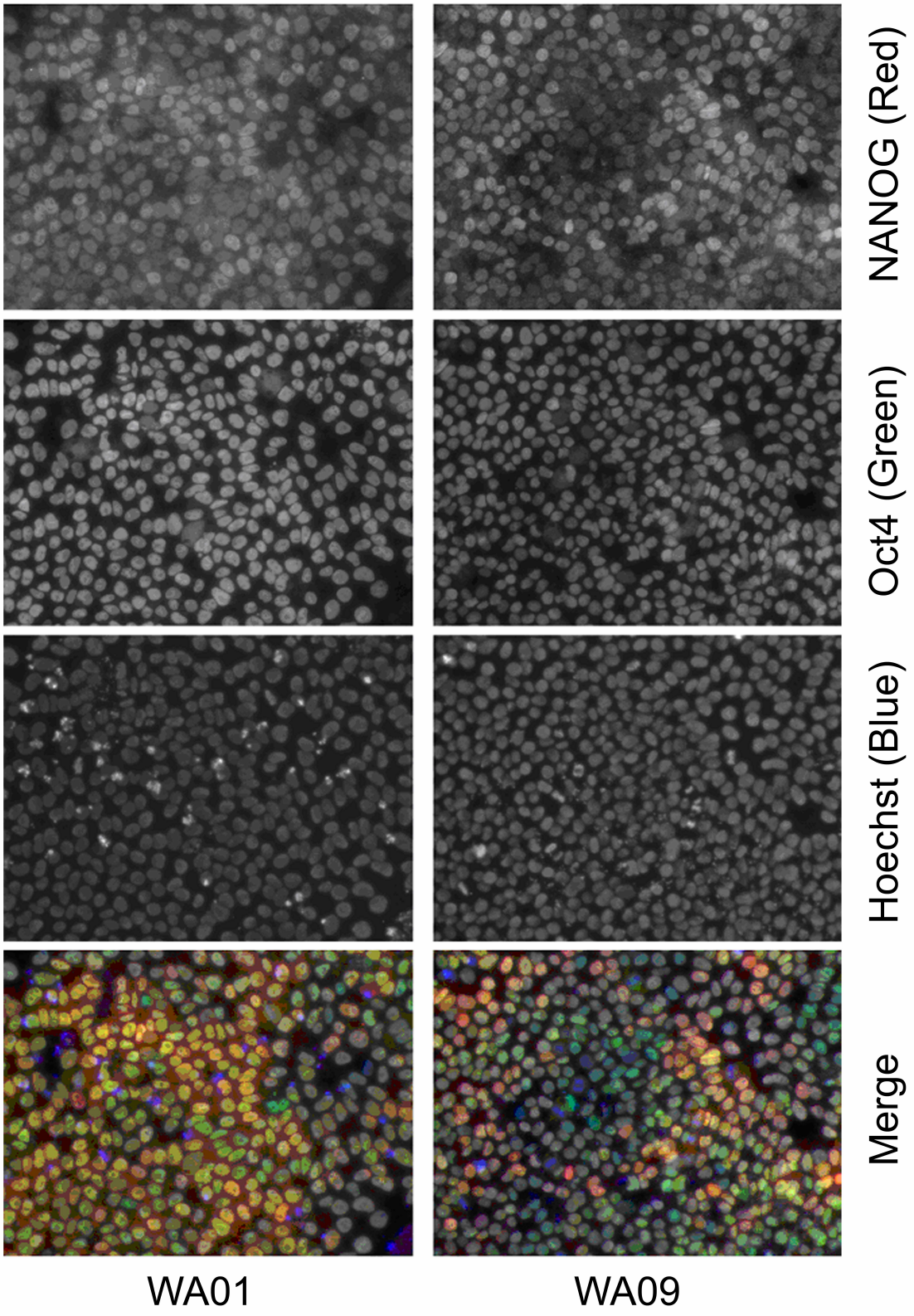
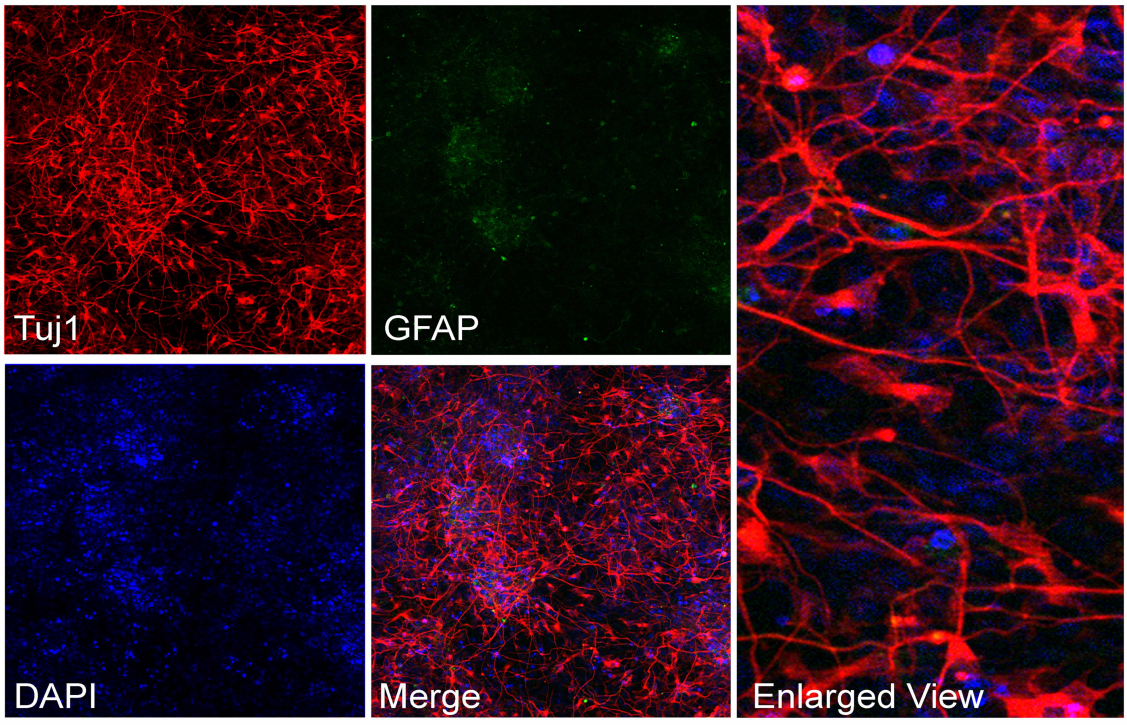
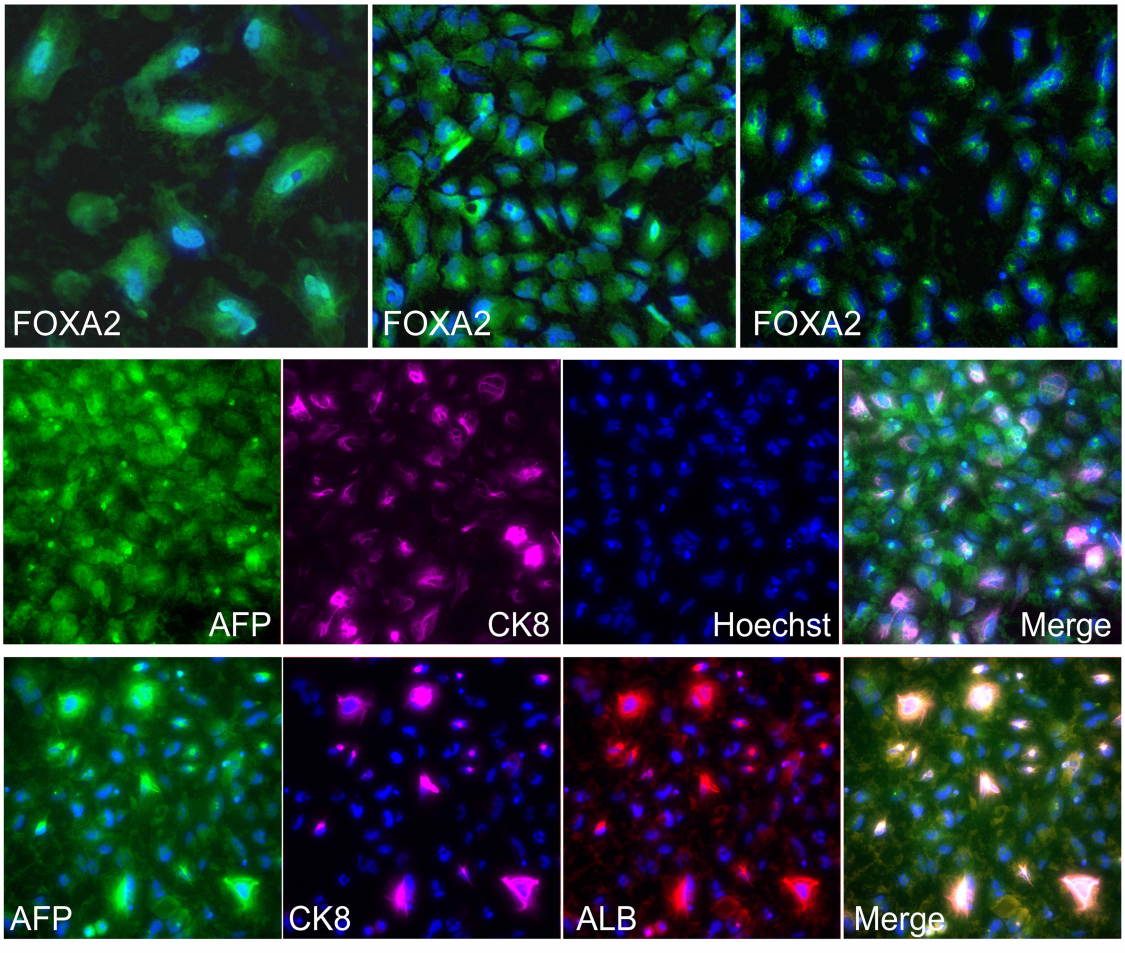


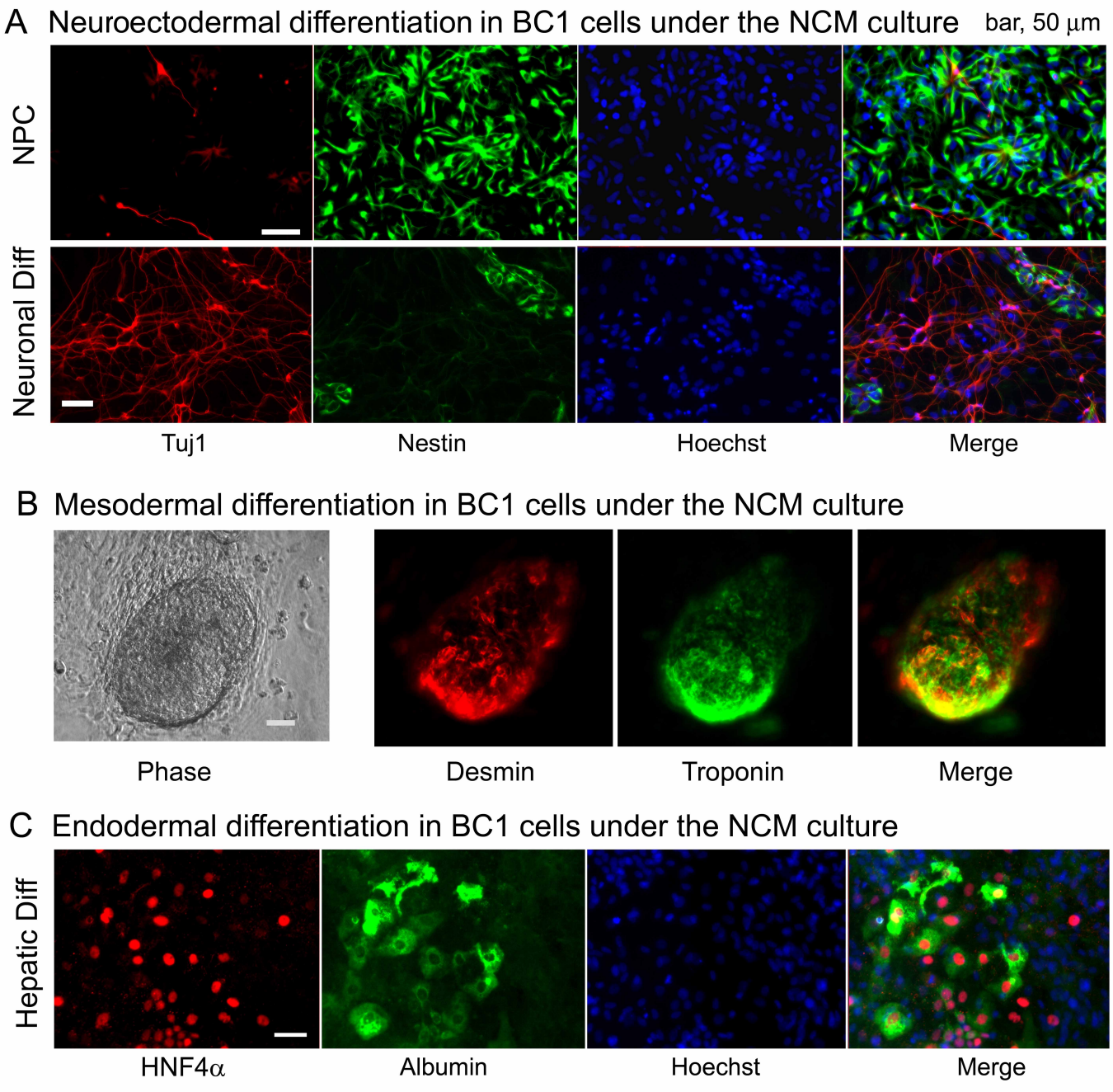
A Neurectoderm



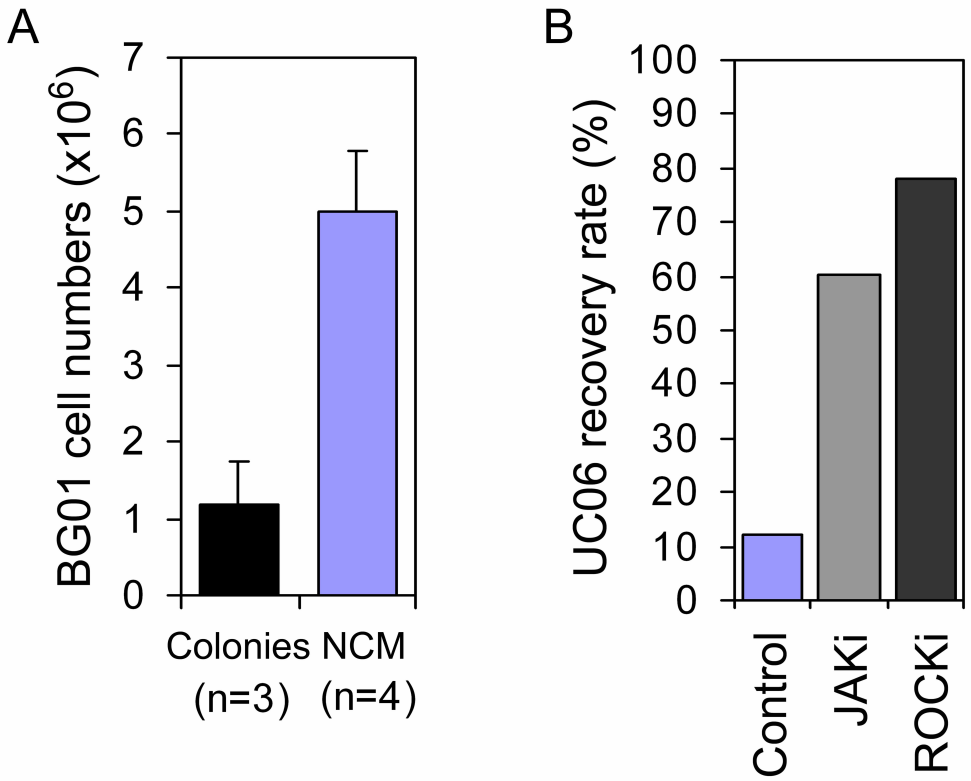
B Mesoderm (hepatic lineages)

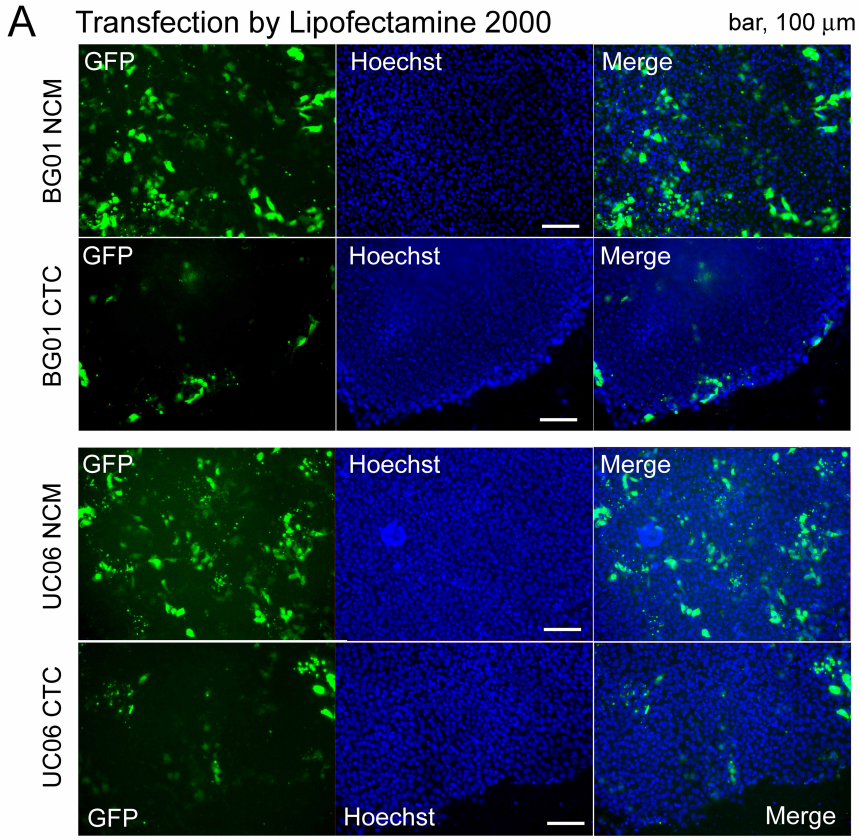


Supplemental Figure 6



Supplemental Figure 7

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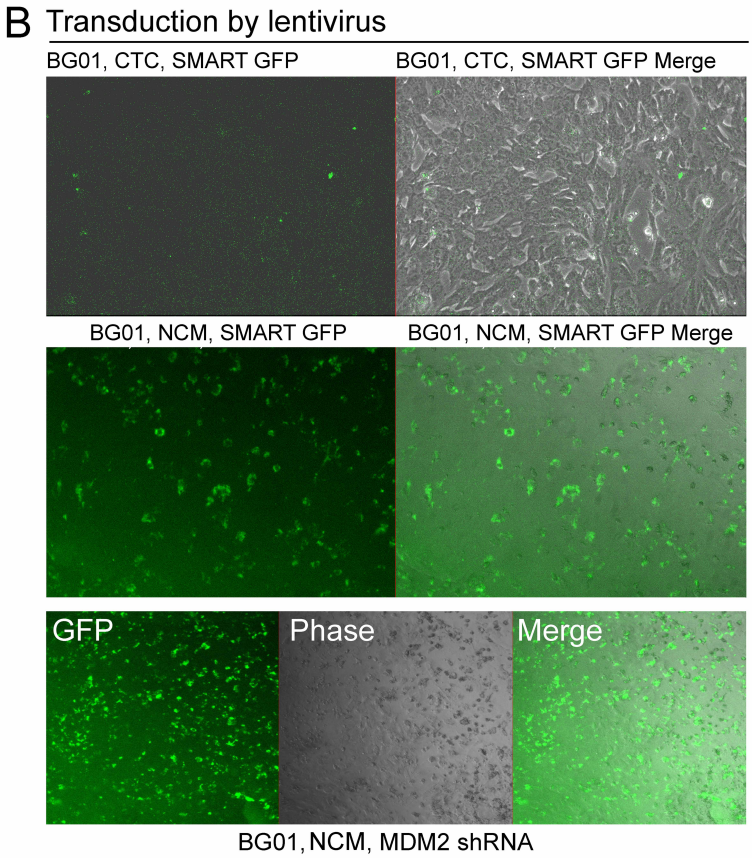
Hoechst
Merge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