Supplemental Materials and Methods

Hepatitis B virus (HBV) infection

The HBV infection was performed according to the protocol described previously (1) with minor modifications. Briefly, the ESC-derived hepatocyte-like cells were cultured with a mixture of serum from patients with chronic HBV infection (HBV viral load = 10^6 copies/ml) and culture medium (1:1, final volume 1 ml) for 3 days. The virus-containing medium was removed, and cells were washed extensively with PBS and then incubated with fresh medium for another 6 days. Afterward, cells were washed with PBS, fixed in 4% paraformaldehyde, and subjected to immunostaining by incubating with polyclonal anti-HBcAg antibody (N1556; Dako, Denmark) and peroxidase-labeled polymers (Dako). Signals were detected using a 3, 3-diaminobenzidine-tetrahydrochloride kit (Sigma). A liver biopsy section from a chronically HBV-infected patient was used as a positive control for HBcAg immunostaining.

Ethoxyresorufin-O-deethylase (EROD) assay

Briefly, 3×10^6 ORMES6 cells with or without hepatic differentiation were grown in medium containing 2 μM of 3-methylcholanthrene for 48 hours. Cells were then re-suspended in 0.25 M potassium phosphate and sonicated. After centrifugation at 9000 × *g* for 20 min at 4℃, 50 μl of supernatant was added to a reaction mixture containing 500 μl HEPES buffer and 500 μl cofactor solution (a mixture of glucose 6-phosphate, glucose 6-phosphate dehydrogenase, $MgCl_2$, and BSA) followed by the addition of 7-ethoxyresorufin and NADPH. After incubating at 37℃ for 10 min, the reactions were terminated and then centrifuged for 10 min at 1,500 × *g*. The degree of conversion of 7-ethoxy to 7-hydroxyresorufin in the supernatant was measured fluorimetrically (550 nm excitation and 585 nm emission).

Isolation of human fibroblasts and iPSC generation

Human fibroblasts were isolated from foreskin and cultured under standard tissue culture conditions. Written informed consent was obtained from the tissue donor, according to the protocol approved by the Internal Research Board (IRB) of National Taiwan University Hospital. Derivation of iPSCs was performed as described previously (2). Briefly, foreskin fibroblasts were plated at a density of 8×10^5 cells/10-cm dish in DMEM with 10% fetal bovine serum. The next day, the lentivirus expressing the retrovirus receptor Slc7a1 (Addgene, Cambridge, MA) and Virapower packaging mix (Invitrogen) were added to the medium of fibroblasts. Selection of fibroblasts expressing Slc7a1 was carried out by blasticidin (12 μg/ml). Plat-E cells (Cell Biolabs, Inc. Japan) were seeded at a density of 8×10^6 cells/10-cm dish with 1 μg/ml puromycin and 10 μg/ml blasticidin and transfected with 9 μg of pMXs-hOct4, pMXs-hSOX2, pMXs-hKlf4, or pMXs-hc-Myc (all from Addgene) using Fugene 6 (Roche). The blasticidin-selected human fibroblasts were transfected with supernatant from 4 different retroviruses (3 ml from each) and polybrene (4 μg/ml). Five days later, the fibroblasts were re-plated onto mitomycin-C-inactivated MEF. The next day, fibroblast medium was replaced by ESC medium, as described above. ES-like cell colonies emerged approximately 20 days after infection. They were picked up manually and cultured in conditions for ESC propagation. All experiments involving recombinant DNA were performed according to the National Institute of Health guidelines.

Characterization of human iPSC cells

To confirm the integration of retroviral transgenes, genomic DNA was extracted from iPSC clones with DNeasy kit (Qiagen, Germantown MD) and PCR analysis was performed with specific primers as described previously (2). For the confirmation of the expression of viral transgenes, Oct4, SOX2, Klf4, Myc, and their endogenous counterparts, total RNA of iPSC clones was extracted using RNaeasy Kit (Qiagen) and RT-PCR was performed with specific primers (2) (Supplementary Table 3).

Teratoma Formation

Approximately 1-2 × 10^6 iPSC cells were injected into the rear leg muscles of 5~8-week-old NOD-SCID mice (National Laboratory Animal Center, Taipei, Taiwan). Teratomas were allowed to develop for 10~12 weeks and were excised and fixed with 4% paraformaldehyde overnight at 4℃ and then transferred to 30% glucose for 48 hours before embedding for cryosectioning. Samples were cut to 10 μm in thickness, transferred to poly-D-lysine-coated slides, and stained with Hematoxylin & Eosin stain (H&E) for histological analysis. All the animal experiments were approved by the Animal Care and Use Committee of Academia Sinica and performed in accordance with the Institutional Animal Care and Use Committee guidelines of Academia Sinica.

Lentivirus-mediated short hairpin RNA interference (RNAi) knockdown

Lentiviral plasmids (PLKO.1) containing different shRNAs driven by the U6 promoter were obtained from the National RNAi Core Facility (Taipei, Taiwan). Different shRNA vectors were delivered into hESdF cells using transient transfection; knockdown efficiency was assayed by QRT-PCR. Clones (clone TRCN0000059267 for INHBA; clone TRCN0000003330 for FGF2) with the best knockdown effect were used for subsequent lentivirus production and infection of hESdF cells using protocols provided by the National RNAi Core Facility (http://rnai.genmed.sinica.edu.tw/Protocols.asp). Infectious lentiviral vectors were harvested at 48 and 72 h post-transfection. Lentiviral infection of hESdF cells was performed using polybren (8 μg/ml, Sigma) and QRT-PCR was performed 72 h after infection to measure the knockdown efficiency.

References:

1. Ochiya, T., Tsurimoto, T., Ueda, K., Okubo, K., Shiozawa, M., and Matsubara, K. (1989) *Proc Natl Acad Sci U S A* **86**, 1875-1879
2. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007) *Cell* **131**, 861-872

Table S1: Antibodies used in this study

Antibodies used in this study	**dilution fold**	**company**	***country***
monoclonal mouse anti-cytokeratin 18	1:50	Invitrogen	*US*
monoclonal mouse anti-human cytokeratin 7	1:50	Dako	*US*
polyclonal rabbit anti-human alpha-1-fetoprotein	1:400	Dako	*US*
monoclonal mouse anti-human cytokeratin 19	1:50	Dako	*US*
polyclonal rabbit anti-human albumin	1:400	Dako	*US*
polyclonal rabbit anti-human cytochrome P450 reductase	1:500	Abcam	*UK*
polyclonal rabbit Anti-HBV core antigen	Ready to use	Dako	*US*
goat polyclonal anti-human Nanog	1:200	R&D	*US*
goat polyclonal anti-human Oct4	1:200	Santa Cruz	*US*
goat polyclonal anti-human brachury	1:50	R&D	*US*
goat polyclonal anti-human Sox1	1:200	Santa cruz	*US*
goat polyclonal anti-human Sox17	1:50	R&D	*US*
Anti-human SSEA4	1:200	Chemicon	*US*
Anti-human Tra1-60	1:200	Chemicon	*US*
Anti-human Sox2	1:100	R&D	*US*

Table S2: Primers used in this study:

Primer ID	sequence(5'-3')	length	Tm	product size
maAFP_S809	GCAGAGGAGACGTGTTGGAT	20	53.8	117
maAFP_AS925	CCAGTGTGGTCAGTTTGCAG	20	53.8	
maTDO2_S956	CGCTTCAGGGAGCATTGAT	19	51.1	147
maTDO2_AS1102	CCAGCATTCTGTGCACCAT	19	51.1	
maCYP7A1_S873	TCCAGCGACTTTCTGGAGTT	20	51.8	122
maCYP7A1_AS994	GATTGCCTTCCAAGCTGACT	20	51.8	
maCYP3A64_S471	GAGAAATCTGAGGCGGGAAG	20	53.8	140
maCYP3A64_AS610	CAAAGGGGTCTTGTGGATTG	20	51.8	
maHNF4A_S311	CAGGCTCAAGAAATGCTTCC	20	51.8	101
maHNF4A_AS411	GGCTGCTGTCCTCATAGCTT	20	53.8	
hAlb_S737	CTCAAGTGTGCCAGTCTCCA	20	60	181
hAlb_AS917	TGGGATTTTTCCAACAGAGG	20	60	
hFGF2_S501	CAAAAACGGGGGCTTCTTCC	20	53.7	150
hFGF2_AS650	GCCAGGTAACGGTTAGCACACAC	23	58.7	
hFGF4_S258	CTACTGCAACGTGGGCATCG	20	55.8	134
hFGF4_AS391	CGCCGAAGATGCTCACCAC	19	55.3	
hINHBA_S284	TCGGGGAGAACGGGTATGTG	20	55.8	126
hINHBA_AS409	GCAGCGTCTTCCTGGCTGTT	20	55.8	
hINHBB_S439	TCATCAGCTTCGCCGAGACA	20	55.8	142
hINHBB_AS580	TCCAGGACGTAGGGCAGGAGT	21	55.8	
hEGF_S627	GCT TGA TAA GCG GCT GTT TTG	22	54.8	146
hEGF_AS772	GGTCACCAAAAAGGGACATTG C	22	54.8	
hVEGF_S566	ATT GGA GCC TTG CCT TGC TG	20	53.8	135
hVEGF_AS700	GAT GGC AGT AGC TGC GCT GA	20	55.9	
hBMP4_S249	GCG GGA TCT TTA CCG GCT TC	20	55.9	149
hBMP4_AS397	TGG TCC CTG GGA TGT TCT CC	20	55.9	
mFGF2_S451	AGGAAGATGGACGGCTGCTG	20	55.9	145
mFGF2_AS590	CTGCCCAGTTCGTTTCAGTGC	21	56.3	
mINHBA_S495	GCAGGAGGGCCGAAATGAAT	20	53.8	146
mINHBA_AS640	CCACACTTCTGCACGCTCCA	20	55.9	
mEGF_S483	GCGAGAGAAGCGGGACTTGT	20	55.9	124
mEGF_AS606	GAGATGCCAGCATCCACCAC	20	55.9	
mVEGF_S1043	GTGCACTGGACCCTGGCTTT	20	55.9	140
mVEGF_AS1182	ATCGGACGGCAGTAGCTTCG	20	55.9	
mBMP4_S729	CAGGGAACCGGGCTTGAGTA	20	55.9	100
mBMP4_AS828	TGG TCC CTG GGA TGT TCT CC	20	55.9	
mNODAL_S498	ACATCATCCGCAGCCTCCA	19	53.2	109
mNODALl_AS606	TCCGCCCATACCAGATCCTC	20	55.9	
maTAT_S49	TGCTTCTTGGAGGCTGCTTTC	21	54.4	150
maTAT_AS198	CATTTTTCCCGGCACAGAGC	20	53.8	
hActB_S15	GAGCACAGAGCCTCGCCTTT	20	55.9	108
hActB_AS122	ACATGCCGGAGCCGTTGTC	19	55.4	
hCK8_S1021	AGATGAACCGGAACATCAGC	20	52.0	120
hCK8_AS1140	CATCCTTAATGGCCAGCTCT	20	52.0	
hCK18_S719	GAGATCGAGGCTCTCAAGGA	20	54.0	124

hCK18_AS842	GAGATTTGGGGGCATCTACC	20	54.0	
hA1AT_S1132	CACCCACGATATCATCACCA	20	52.0	146
hA1AT_AS1277	CCCCATTGCTGAAGACCTTA	20	52.0	
mFGF4_S308	CTACTGCAACGTGGGCATCG	20	55.8	198
mFGF4-AS505	CTGGCCACTCCGAAGATGCT	20	55.9	
hAQP1_S396	AGGCATCACCTCCTCCCTGA	20	56.0	189
hAQP1_AS584	ACAGAGAGGCCGATGGCAAG	20	58.0	
hKRT19_S469	GGTGAAGATCCGCGACTGGT	20	56.0	200
hKRT19_AS668	GCAGAGCCTGTTCCGTCTCA	20	56.0	

Supplemental figure legends

Supplemental Figure S1.

Phase-contrast images of monkey ESCs (ORMES6) co-cultured with ESdFs for 2 months. The differentiated cells had clear round nuclei with densely granulated or vacuolated cytoplasm, which are characteristics of mature hepatocytes. Scale bar, 25 μm

Supplemental Figure S2.

Comparsion of endodermal-to-hepatic differentiation among individual primate ESC colonies treated with hESdFs. The gene expression level of the endodermal and hepatic marker genes in single cell colonies of both NTU1 and OREMS6 at IVDS1, 2 and 3 were analyzed by QRT-PCR. Box-and-whisker plots (each box plot indicates gene expression of 7 individual colonies) of GATA4 (endoderm), CK19 (early hepatocytes), AFP (early hepatocytes) and albumin (mature hepatocytes). The top, bottom, and inner line of each box plot indicates the 75th percentile (top quartile), 25th percentile (bottom quartile), and 50th percentile (median), respectively, of gene expression level among the 7 colonies. Grubbs test did not detect any outliers from these values, indicating that no expression value lay outside the overall pattern of distribution. (All values of $Z < N-1/\sqrt{N}$; no P values are less than 0.05)

Supplemental Figure S3.

Human hESdFs derived from different hESC lines promote hepatic differentiation in multiple primate ESC lines. (A) Human H9- or (B) NTU1-derived hESdFs were co-cultured with two hESC lines, NTU1 and NTU3, for HLC formation. The hepatic gene expression of NTU1-derived HLCs and NTU3-derived HLCs were analyzed by RT-PCR. ICC analysis of hepatocyte marker expression in NTU3-derived HLCs showed that the majority of the HLCs were positive for the indicated hepatic markers. Nuclei were stained with DAPI (blue). Scale bar, 25 μm.

Supplemental Figure S4.

hESdFs promote cholangiocyte differentiation in human and monkey ESCs. (A) QRT-PCR analysis was applied to IVDS 1, 2, and 3 with cholangiocyte genes CK19 and AQP1; albumin gene expression was used as an index to show the degree of HLC formation. (B) IVDS3 HLC foci derived from monkey ESCs (ORMES6) and human ESCs (NTU1) were partially positive for CK19. Nuclei were stained with DAPI (blue). Scale bar, 25 μm.

Supplemental Figure S5.

Production of albumin in human ESC derived HLCs under hESdF co-culture. ELISA assay showed albumin production in the differentiated human ESCs (NTU1) at different differentiation time points. Significant differences between each time point and ESC media are labeled. (*$P < 0.05$)

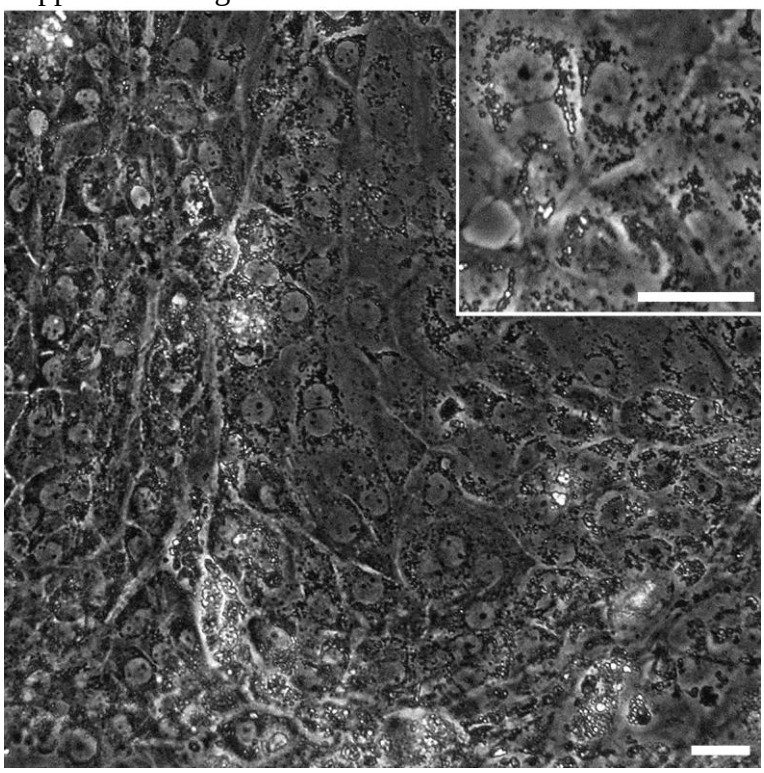
Supplemental Figure S6.

Comparison of albumin production from human and monkey HLCs generated by EB-mediated spontaneous differentiation, hESdF co-culture and 3-step methods. (A) ELISA assay of albumin level of human HLCs generated by 3 different differentiation methods. (B) Bromocresol purple assay of albumin level of monkey HLCs generated by 3 different differentiation methods. The hESdF co-culture and 3-step methods were equally competent at promoting albumin production from both human (A) and monkey (B) HLCs, while human HLCs generated by the EB-based method produced less albumin than the other two methods (*$P < 0.05$)

Supplemental Figure S7.

Derivation and characterization of human iPSC clones. (A) PCR analysis (upper panel) of retroviral integration and transgenic expression using genomic DNA isolated from putative iPSC clones with transgene-specific primers. RT-PCR analysis (lower panel) of the expression of retroviral transgenic and endogenous Oct4, SOX2, KLF4, and C-MYC in the putative iPSC clones and hESC H9. Note that the silencing of all 4 transgenes and the reactivation of their endogenous counterparts were found in 3 clones (clones 10, 37, and 50, labeled with *). (B) A phase-contrast image (upper left panel) of the colony from a representative human iPSC, clone 37, and immunostaining of human iPSCs with antibodies against human ESC-related markers including Oct4, NANOG, SSEA4, TRA-1-60, and SOX2. Nuclei were counterstained with DAPI (blue). Scale bar, 25 μm (C) H&E staining of the teratoma from the *in vivo* differentiation of iPSC clone 37 in SCID mice showed differentiated derivatives of ectodermal, mesodermal, and endodermal lineages.

Supplemental Fig. S1.



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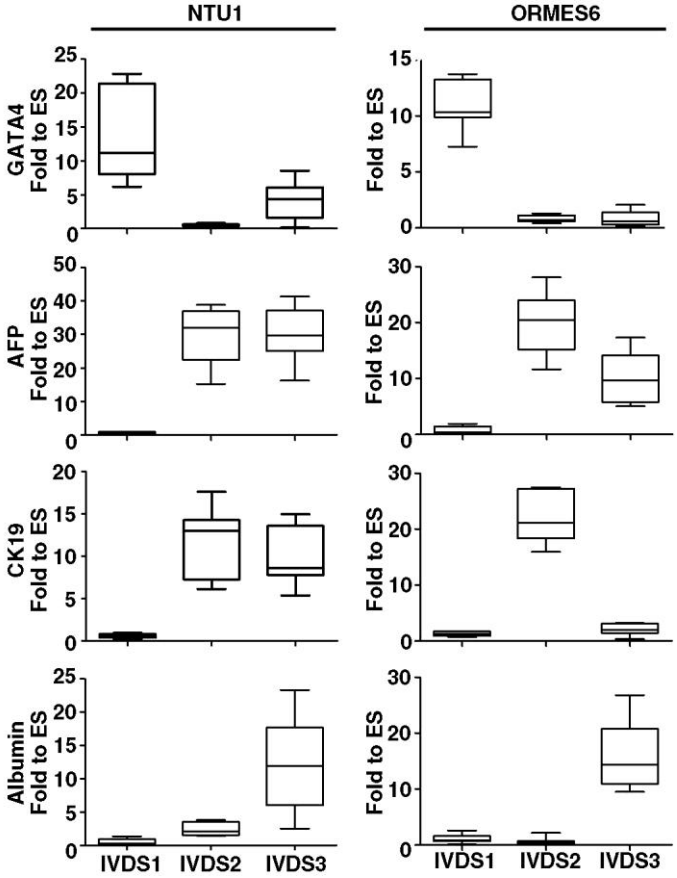

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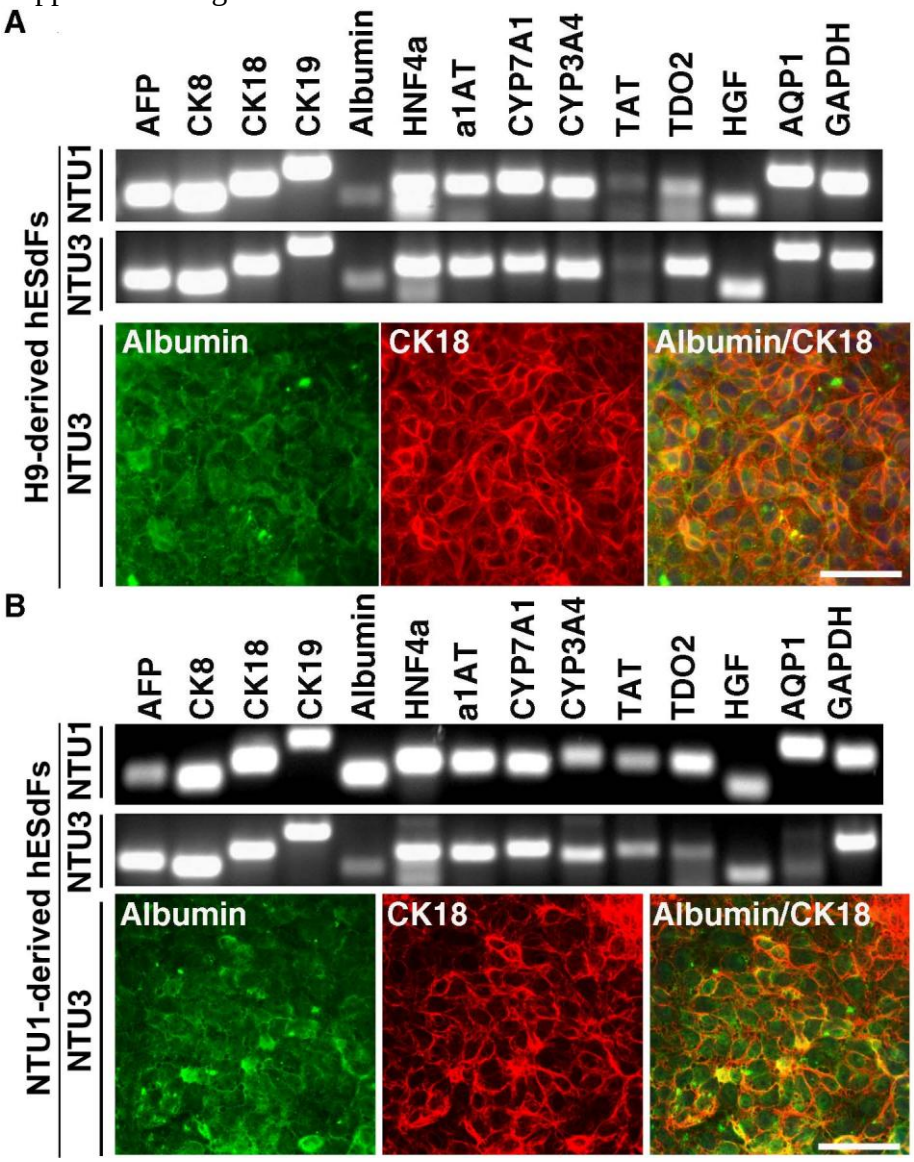

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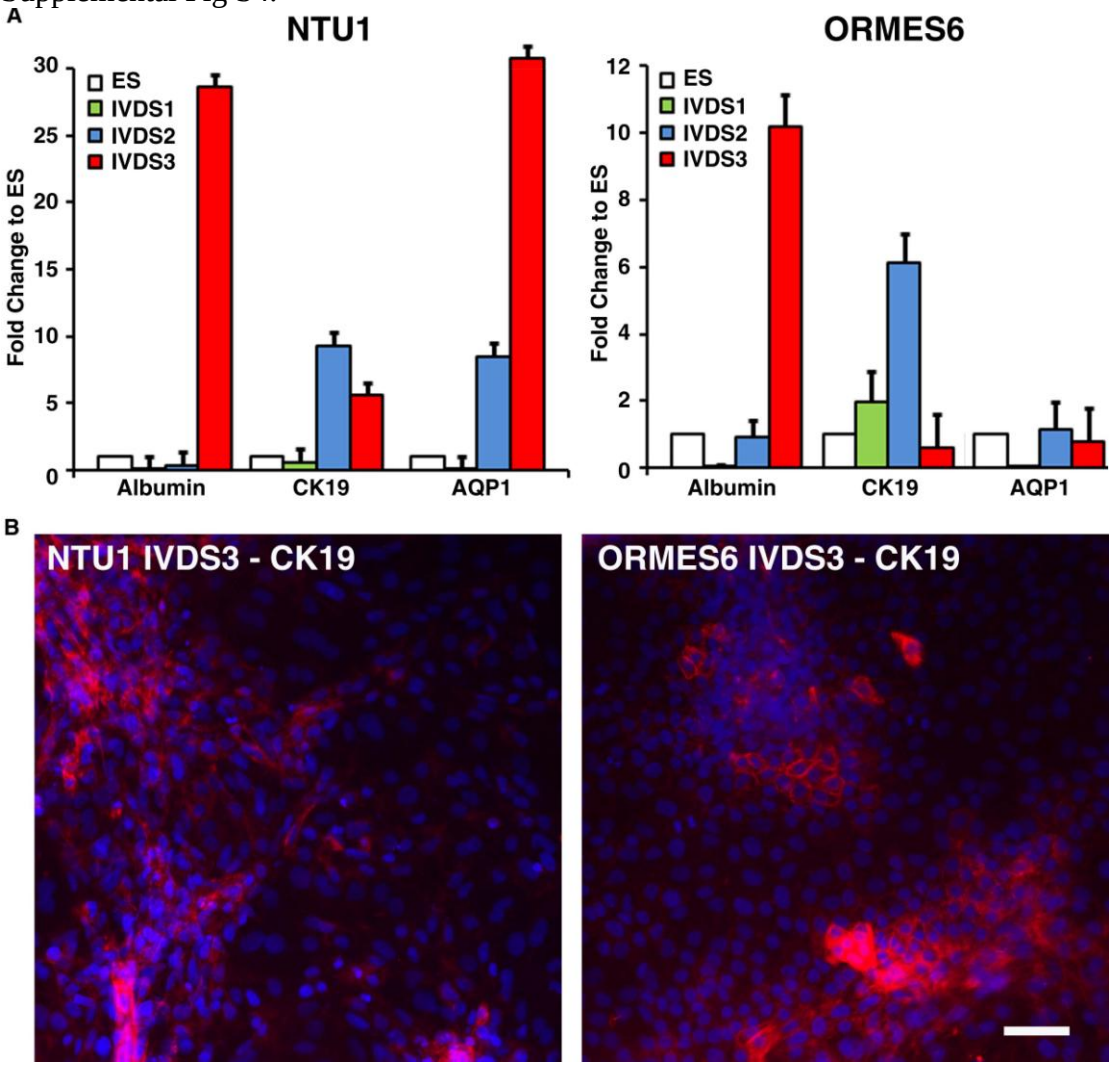

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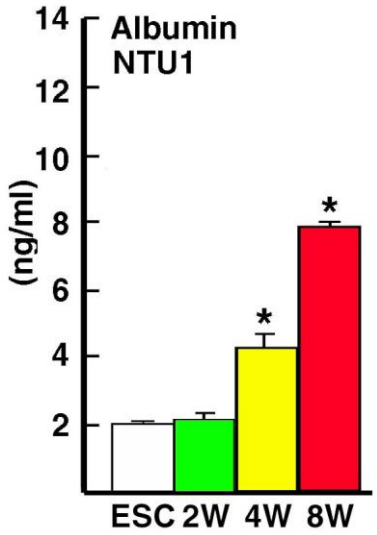

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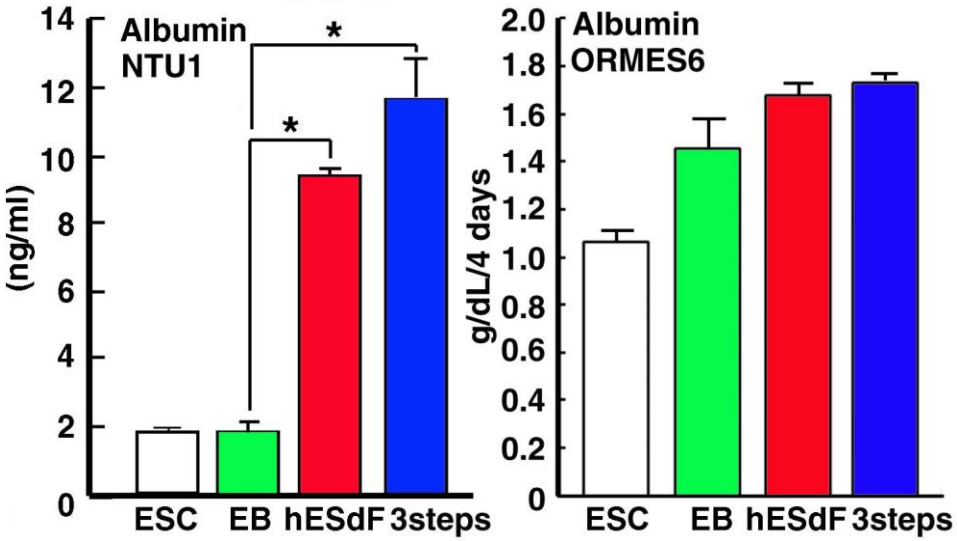

Supplemental Fig S7.