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Supplemental Information

Endoderm Generates Endothelial Cells during Liver Development

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SUPPLEMENTAL FIGURES AND LEGENDS

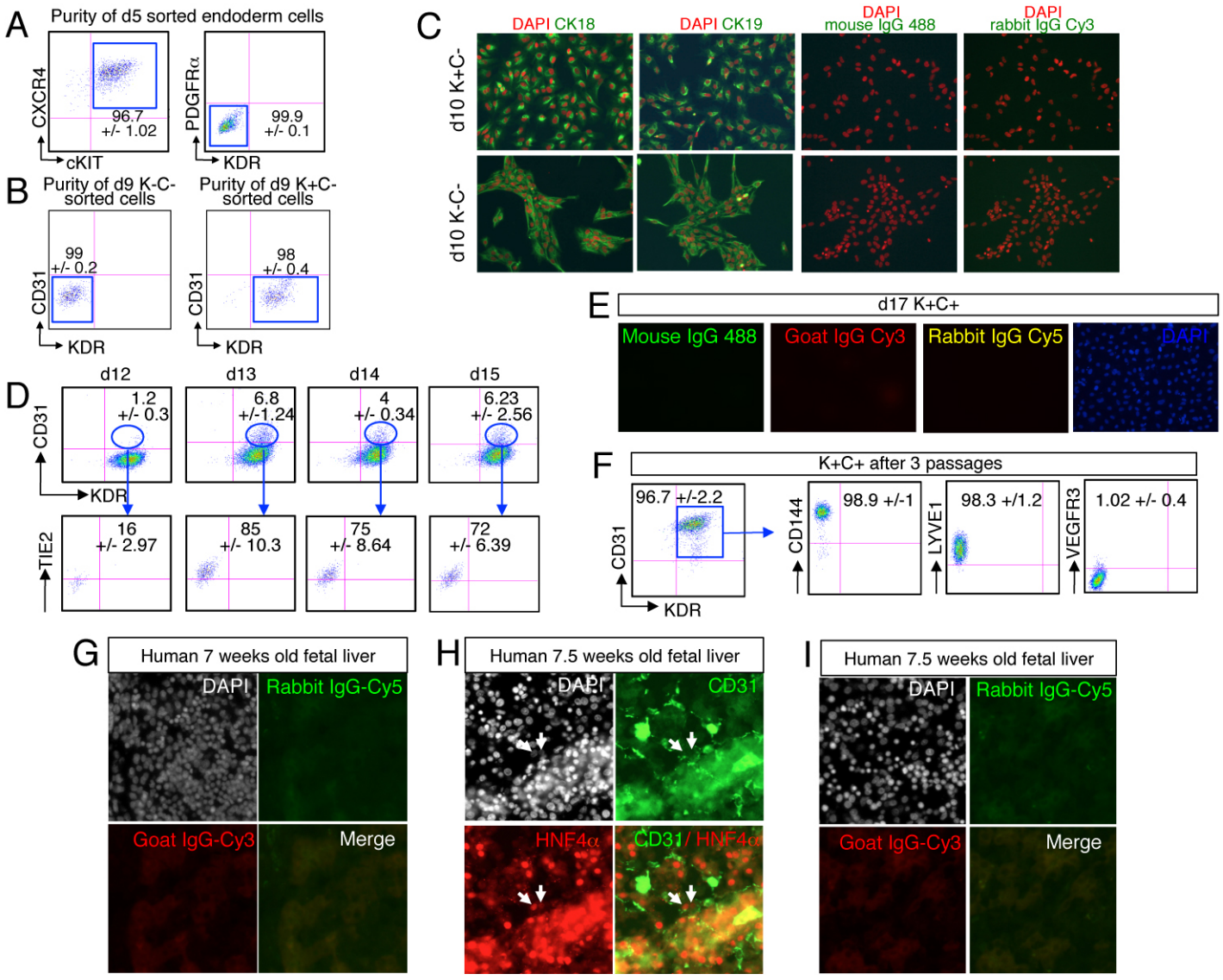

Figure S1 for Figure 1: Characterization of ECs from human ESC-derived KDR+ endoderm cells and human fetal livers.

(A) Representative flow cytometry analyses of the purity of day 5 endoderm populations isolated within the CXCR4+cKIT+KDR-PDGFRα- fraction, and (B) of the K+C- and K-C- populations isolated from day 9 hepatic cultures. Numbers represent means +/- SD of n=10 independent experiments. (C) Immunostaining using antibodies against CK18 and CK19, and IgG controls of K+C- and K-C- populations purified at day 9 and cultured for 1 day (day10 of differentiation) in hepatic conditions (x200). (D) Flow cytometry analysis of day 12 to day 15 cultures obtained from day 9 isolated K+C- cells (n=3). (E) IgG controls for Figure 1H (x200). (F) Flow cytometry analysis from purified K+C+ cells after 3 passages (n=3). (G) IgG controls for Figure 1J. (x200). (H) Co-immunostainings for CD31 and HNF4α (x200), (I) and IgG controls (x200) on 7.5 weeks old human fetal liver sections.

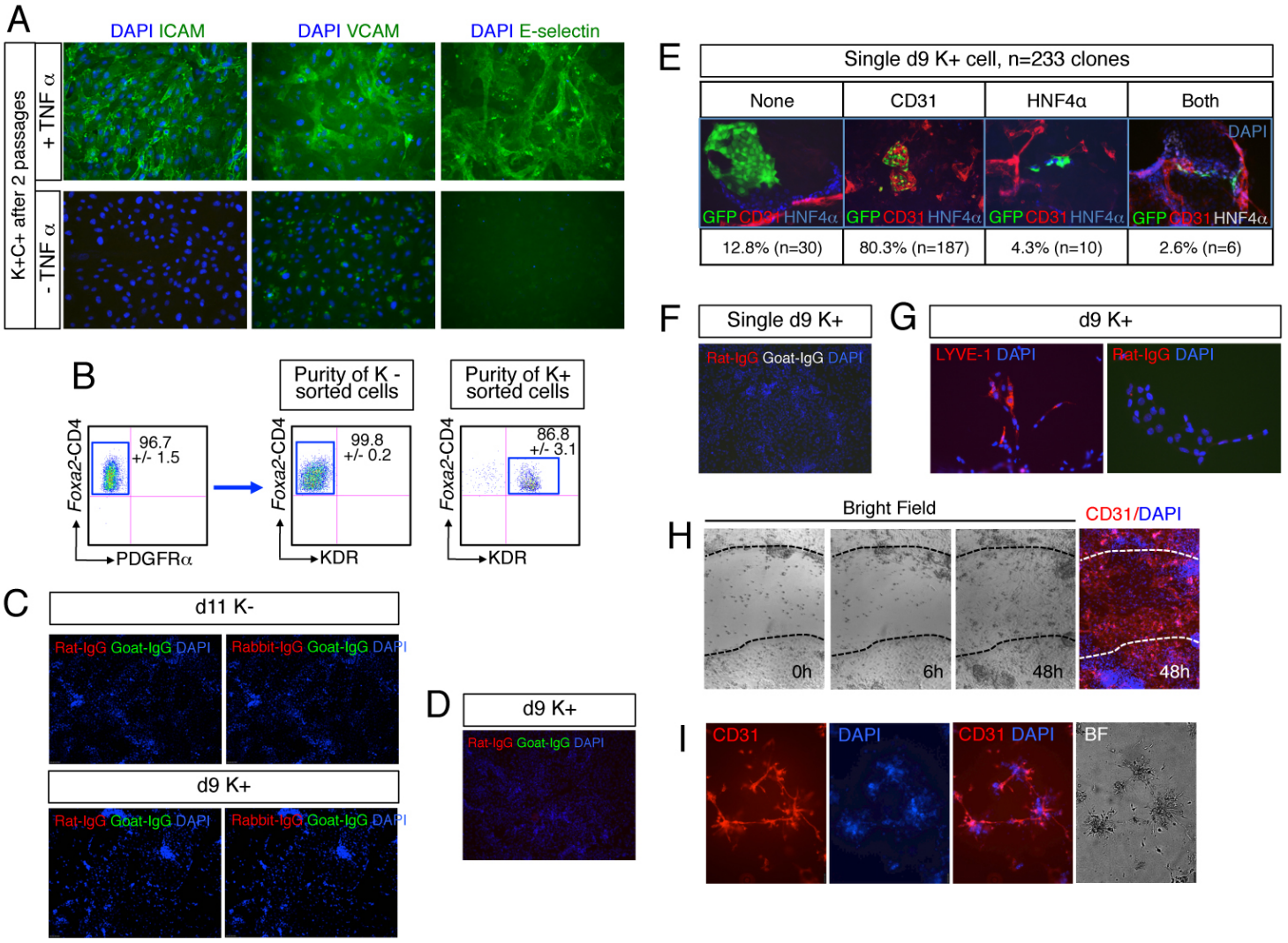

Figure S2 for Figure 2 and 3: Characterization of ECs from human and mouse ESC-derived KDR+ endoderm cells.

(A) Immunostaining of K+C+ cells after 2 passages using antibodies against ICAM, VCAM and E-selectin following TNFα activation (x200). (B) Representative flow cytometry analyses for the purity of day 6 K+ and K- populations isolated within the *Foxa2*-CD4+*T*-GFP-PDGFRα-KDR+ (named K+ cells) or *FoxA2*-CD4+*T*-GFP-PDGFRα-KDR- (named K- cells) fractions respectively. Numbers represent means +/- SD of n=3 independent experiments. (C) IgG controls counterstained with DAPI of immunostaining shown in Figure 3B (x200). (D) IgG control for immunostaining of Figure 3D (X200). (E) Summary of the cell fate of 233 clones generated in clonal assay of K+ cells at day 9 of differentiation (X200). (F) IgG control for immunostaining of the clonal assay (X100). (G) LYVE1 immunostaining and IgG control on day 9 K+ cells (X200). (H) Wound healing assay of K+ cells. Migrating endothelial cells were identified following immunostaining for CD31 in the dish (x200). (I) Tube formation assay in matrigel by K+ cells. Immunostaining for CD31 of K+ cells was also performed (x200).

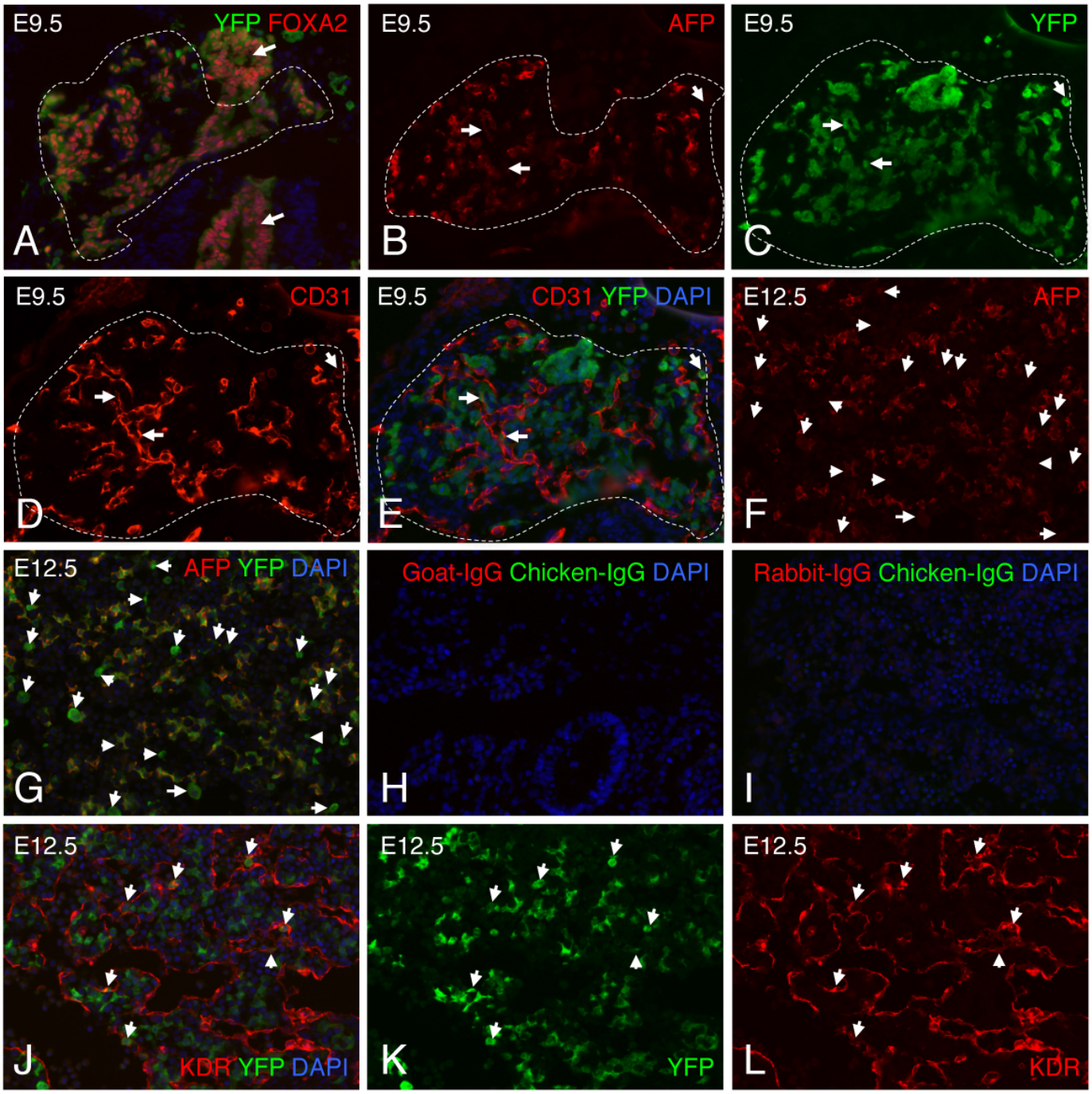

Figure S3 for Figure 4: *Foxa2*-iCre lineage tracing mouse analyses.

(A-E) Immunostaining of E9.5 embryo sections of *Foxa2*-iCre;YFP mice for YFP, FOXA2, AFP and CD31 (A: x100, B-E: X200). (B) represents the liver bud on the consecutive section from (A). (C-E) represent the same field of the liver bud on the same section with single (C, D) or merged staining (E) on the following consecutive section from (B). (F-G) Immunostaining of the same E12.5 embryo fetal liver section shown in Figure 4A-C for YFP and AFP indicating that all CD31+YFP+ cells (arrows) are negative for AFP (x200). (H-I) Immunostainings with IgGs controls for KDR, CD31 and YFP antibodies on E12.5 fetal liver sections. (J-L) Immunostaining for YFP, KDR and DAPI of a consecutive section from the E12.5 embryo shown in Figure 4A-C (X200). Arrows indicate KDR+YFP+ cells.

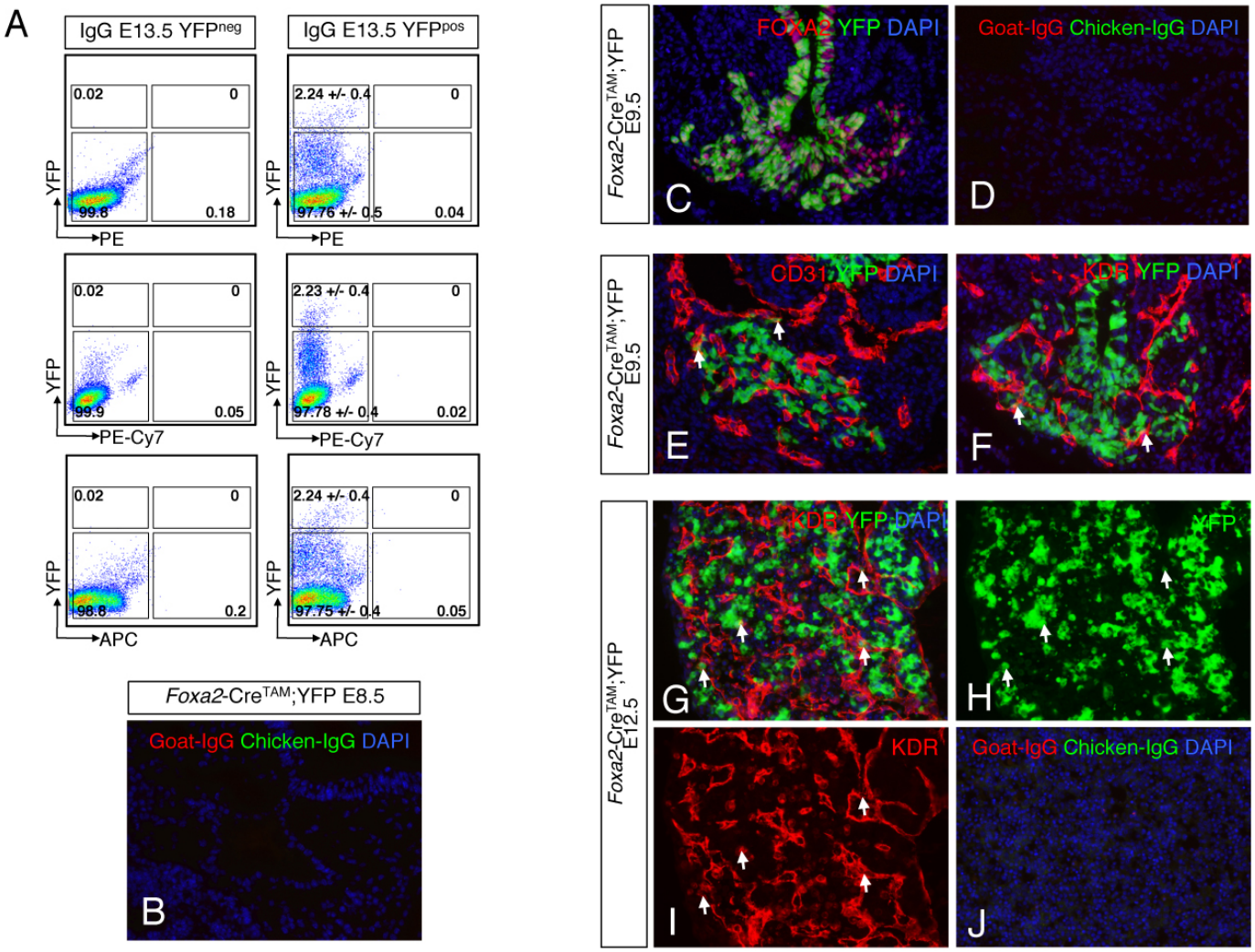

Figure S4 for Figure 4: *Foxa2*-iCre and *Foxa2*-Cre^{TAM} lineage tracing mouse model analyses.

(A) IgG controls for flow cytometry analyses of E13.5 fetal liver cells from *Foxa2*-iCre;YFP mice. Numbers indicate the means +/- SD of the percentage of cells in the respective gates for n=4 embryos YFP^{neg} and 1 embryo YFP^{pos}. (B) IgG controls for immunostaining of E8.5 *Foxa2*-Cre^{TAM};YFP embryos for Figure 4E. (C-D) FOXA2 and YFP immunostaining and IgG controls of E9.5 liver bud section of *Foxa2*-Cre^{TAM};YFP mice validating the high specificity of YFP mapping for FOXA2+ endoderm cells and hepatoblasts (x200). (E-F) Co-immunostaining for YFP and CD31 (E) or KDR (F) on liver bud section at E9.5 of *Foxa2*-Cre^{TAM};YFP mice showing rare single endothelial cells originated from FOXA2+ endoderm progenitors (X200). (G-J) YFP and KDR immunostaining and IgG controls on E12.5 liver section of *Foxa2*-Cre^{TAM};YFP mice showing cells co-expressing YFP and KDR (arrows, X200).

SUPPLEMENTAL TABLES

Antibody	Species	Company	Cat. Number	Application	Dilution
CXCR4-PE	Mouse	R&D System	FAB170P	FC	1/20
cKit-PECy7	Mouse	BD Biosciences	339206	FC	1/20
PDGF-FITC	Mouse	Caltag	K0148-4	FC	1/20
KDR-647	Mouse	BD Biosciences	560495	FC	1/20
CD31-FITC	Mouse	BD Biosciences	555445	FC	1/100
CD144-PE	Mouse	Beckman Coulter	A07481	FC	1/20
TIE2-PE	Mouse	millipore	FCMAB404PE	FC	1/100
ICAM	Mouse	R&D System	BBA3	FC	1/20
VCAM	Mouse	R&D System	BBA5	FC	1/20
CK18	Mouse	Sigma	C8541	ICC	1/100
CK19	Mouse	Dako	M0888	ICC	1/100
HNF4α	Goat	SantaCruz	Sc-6556	ICC	1/100
Mouse IgG		Jackson Immunoresearch laboratories	015-000-003	FC	
CD31	mouse	BD Biosciences	555675	ICC	1/100
KDR	Goat	R&D System	AF357	ICC	1/20
CD31	Rabbit	invitrogen	550389	IHC	1/100
vWF	rabbit	Dako	A0082	ICC	1/700
Goat IgG		Jackson Immunoresearch laboratories	005-000-003	ICC IHC	
Rabbit IgG		Jackson Immunoresearch laboratories	011-000-003	ICC IHC	
Donkey anti mouse 488		Jackson Immunoresearch laboratories	A21202	ICC IHC	1/250
Donkey anti goat Cy3		Jackson Immunoresearch laboratories	705-165-147	ICC IHC	1/250
Donkey anti mouse Cy3		Jackson Immunoresearch laboratories	715-165-150	ICC IHC	1/250
Donkey anti rabbit Cy5		Jackson Immunoresearch laboratories	711-606-152	ICC IHC	1/250

Table S1: Supplemental experimental procedures, antibodies against human proteins used in Figures 1, 2 and S1.

Antibody	Species	Company	Cat. Number	Application	Dilution
hCD4-APC	Mouse	Invitrogen	MHCD0405	FC	1/100
PDGFRα-PE	Rat	eBioscience	12-1401-81	FC	1/100
KDR-PECy7	Rat	BD Biosciences	561259	FC	1/150
CD31-APC	Rat	BD Biosciences	551262	FC	1/100
TIE2-PE	Rat	eBioscience	12-5987-81	FC	1/100
CD144-APC	Rat	eBioscience	17-1441-80	FC	1/100
DLK-PE	Rat	MBL	D187-5	FC	1/100
ICAM-APC	Rat	R&D Systems	FAB796A	FC	1/100
FOXA2	Goat	Santa cruz	Sc-6554	ICC IHC	1/50
HNF4α	Goat	Santa cruz	Sc-6556	ICC	1/100
AFP	Rabbit	Neomarkers	RB-365-A	ICC IHC	1/200
KDR	Goat	R&D Systems	AF644	IHC	1/30
CD31	Goat	R&D Systems	AF3628	IHC	1/30
CD31	Rat	BD	557355	ICC	1/150
LYVE1	Rat	Santa cruz	Sc-65647	ICC	1/50
GFP (YFP)	Chicken	Invitrogen	A10262	IHC	1/300
Rat IgG		Jackson Immunoresearch laboratories	012-000-003	ICC	
Goat IgG		Jackson Immunoresearch laboratories	005-000-003	ICC IHC	
Rabbit IgG		Jackson Immunoresearch laboratories	011-000-003	ICC IHC	
Chicken IgG-Y		Jackson Immunoresearch laboratories	003-000-003	IHC	
Donkey anti chicken Cy3		Jackson Immunoresearch laboratories	703-162-155	IHC	1/400
Donkey anti goat A647		Jackson Immunoresearch laboratories	705-605-147	IHC	1/400
Donkey anti goat A488		Invitrogen	A11055	ICC	1/400
Donkey anti rabbit A647		Jackson Immunoresearch laboratories	711-606-152	ICC IHC	1/400
Donkey anti rat cy3		Jackson Immunoresearch laboratories	712-166-153	ICC	1/400

Table S2: Supplemental experimental procedures, antibodies against mouse proteins used in Figures 3, 4, S2, S3 and S4.

Name	Forward	Reverse
β-ACTIN	5'- TTTTTGGCTTGACTCAGGATTT-3'	5'- GCAAGGGACTTCCTGTAACAAC-3'
LYVE-1	5'- TGGGGATCACCCTTGTGAG-3'	5'- AGCCATAGCTGCAAGTTTCAAA-3'
VEGFR3	5'-TGCACGAGGTACATGCCAAC-3'	5'-GCTGCTCAAAGTCTCTCACGAA-3'
HNF4α	5'-CACGGGCAAACACTACGGT-3'	5'-TTGACCTTCGAGTGCTGATCC-3'

Table S3: Supplemental experimental procedures, list of human primers for qPCR used in Figure 1.

Name	Forward	Reverse
β-Actin	5'-TGAGCGCAAGTACTCTGTGTGGAT-3'	5'-ACTCATCGTACTCCTGCTTGCTGA-3'
Foxa2	5'-CTCTCTCTCCTTCAACGACTGCTTTCTC-3'	5'-TTCCTTCAGTGCCAGTTGCTTCTC-3'
EpCAM	5'-CGTGAGGACCTACTGGATCAT-3'	5'-GTCCACGTCGTCTTGTGTTTT-3'
HNF4α	5'- AAGGTGCCAACCTCAATTCATC -3'	5'- CACATTGTCGGCTAAACCTGC -3'
Afp	5'-GGAATGAAGCAAGCklkCCTGTGAACT -3'	5'-AACTGGTGATGCATAGCCTCCTGT-3'
Kdr	5'-TTTGGCAAATACAACCCTTCAGA-3'	5'-GCTCCAGTATCATTTCCAACCA-3'
CD31	5'-TGAAGACAGACCTCAAGCCAGCAA-3'	5'-ACAGCTCAGACCTTAGGAAACCGT-3'
CD144	5'-GTCGATGCTAACACAGGGAATG-3'	5'-AATACCTGGTGCGAAAACACA-3'
Lyve1	5'-CAGCACACTAGCCTGGTGTTA-3'	5'-CGCCCATGATTCTGCATGTAGA-3'
Vegfr3	5'-GGCAAATGGTTACTCCATGACC-3'	5'-ACAACCCGTGTGTCTTCACTG-3'
Stabilin	5'-AGCTGCTGCCTTTAATCCTCA-3'	5'-ACTCCGTCTTGATGGTTAGAGTA-3'

Table S4: Supplemental experimental procedures, list of mouse primers for qPCR used in Figure 3.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Flow cytometry analyses

Cultured cells were dissociated with trypsin/EDTA or E13.5 fetal liver embryos were dissociated as previously described (Goldman et al., 2013). Cells were then immunostained with specific antibody (see Tables S1, S2) in PBS with BSA 0.1% at room temperature for 20 minutes. Cells were then analyzed using a LSRII flow cytometer (Becton Dickinson). Fluorochrome-labeled IgGs were used as controls for human CD31, VCAM, ICAM, E-selectin, Tie2, CD144, LYVE1 and VEGFR3 stainings. Absence of antibody was otherwise considered as control stainings as positive stainings were far away from the negative gate.

Wound healing assay

Cells were expended in hepatic media. A starch was made with a cell culture tip and pictures were taken at different time points following further culture.

Uptake of acetylated LDL

Cells were incubated for 6 hours with 15 ng/ml of LDL-ac-GFP (life sciences). Stained cells were visualized using a fluorescent microscope Leica.

Vascular tube formation

250,000 human cells were seeded in a 12-well plate or 20,000 mouse cells were seeded in a 48-well plate coated with matrigel in EGM2 media (Lonza) with 50 ng/ml of VEGF (R&D). Pictures were taken 24 hours after seeding with a microscope Leica.

Tumor necrosis factor assay

Cells were incubated during 18 hours with 10 ng/ml of TNFα (R&D system). Cells were then dissociated with trypsine/EDTA and immunostained with antibodies against ICAM or VCAM for 20minutes.

Clonal assay

Ef1α- GFP lentivirus tagged mouse ESC line was differentiated into definitive endoderm similarly to the *T*-GFP/*Foxa2*-CD4 ESCs (Gift from Dr. Darrell Kotton, Boston University). At day 6 of differentiation, single alive GFP+FOXA2+KDR+PDGFRα- cells were isolated and cultured with 8,000 non-GFP-tagged day 6 differentiated ESCs (*T*-GFP/*Foxa2*-CD4) in 96 well plates in hepatic medium as previously described (Han et al., 2011). Hepatic endoderm and endothelial fate of the GFP+ clones was determined by HNF4α and CD31 immunostaining, respectively, 3 days after plating.

In vivo ischemia assay

Adult NOD/SCID mice were injected with 50μl of 0.25 x10^6 human K+C- cells into 4 different points of a muscle in the area where the femoral artery was ligated as previously described (Vanneaux et al., 2010). Seven days after transplantation, 100μl GFP-labeled dextran (25mg/4ml; Life Technologies, NY) was injected through the carotid artery prior to sacrifice.

Immunostainings

Adherent cells were fixed with 4% paraformaldehyde for 15 min and incubated for 30 minutes with Dako blocking buffer. Cells were permeabilized with Triton X-100 0.3%, incubated for 30 minutes with the Dako blocking buffer and stained with primary antibodies overnight at 4°C. The next morning, cells were then washed and incubated with secondary antibodies for 1 hour at room temperature and counterstained with DAPI. For co-staining with CD31, cells were first immunostained with CD31 overnight and then permeabilized prior to the second immunostaining (see Table S1 and S2 for the list of antibodies).

Immunohistochemistry

E9.5 and E12.5 embryos were fixed for 2 hours with 4% PFA at 4°C, while muscle tissues or human fetal livers were fixed over night. Tissues were then washed with PBS for 3 times and dehydrated with 30% sucrose and embedded in OCT. Cryosections of 8-µm were blocked with 3% of donkey serum with or without permeabilization (for CD31 staining) in the presence of 0.3% Triton X-100, immunostained with primary antibodies (see Table S1 and S2) overnight at 4°C. Sections were then incubated for 1 hour with secondary antibodies (see Table S1 and S2) at room temperature and counterstained with DAPI. Sections were visualized using a fluorescent confocal microscope (Leica).

RNA extraction and qPCR

Total RNA was prepared with the RNeasy micro kit (Qiagen). RNA was reverse transcribed into cDNA using the Super Script-III First-strand Synthesis System kit (Invitrogen). qPCR was performed with the Roche system LC480. All experiments were done in triplicate using the Roche SYBR Green master mix. Relative quantification was calculated using the comparative threshold cycle (CT) method and was normalized against the ΔCT of the housekeeping gene β-actin. Melting curves for each gene were used to confirm homogeneity of the DNA product (see the list of primers in Table S3 and S4).

Statistic analyses

All numbers reflect means +/- SD for n independent experiments. Calculations were performed with the paired t-tests. Significance were defined as * $p< 0.05$, ** $p<0.01$, and *** $p<0.001$***.

SUPPLEMENTAL REFERENCES

Vanneaux, V., El-Ayoubi, F., Delmau, C., Driancourt, C., Lecourt, S., Grelier, A., Cras, A., Cuccuini, W., Soulier, J., Lataillade, J.J., et al. (2010). In vitro and in vivo analysis of endothelial progenitor cells from cryopreserved umbilical cord blood: are we ready for clinical application? Cell Transplant 19, 1143-1155.