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Endothelial cells instruct liver specification of embryonic stem cell-derived endoderm through endothelial VEGFR2 signaling and endoderm epigenetic modifications

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ABSTRACT

Liver organogenesis requires complex cell-cell interactions between hepatic endoderm cells and adjacent cell niches. Endothelial cells are key players for endoderm hepatic fate decision. We previously demonstrated that the endothelial cell niche promotes hepatic specification of mouse embryonic stem cell(ESC)-derived endoderm through dual repression of Wnt and Notch pathways in endoderm cells. In the present study, we dissected further the mechanisms by which endothelial cells trigger endoderm hepatic specification. Using our previously established in vitro mouse ESC system mimicking the early hepatic specification process, endoderm cells were purified and co-cultured with endothelial cells to induce hepatic specification. The comparison of transcriptome profiles between hepatic endoderm cells isolated from co-cultures and endoderm cells cultured alone revealed that VEGF signaling instructs hepatic specification of endoderm cells through endothelial VEGFR2 activation. Additionally, epigenetic mark inhibition assays upon co-cultures uncovered that histone acetylation and DNA methylation promote hepatic specification while histone methylation inhibits it. This study provides an efficient 2D platform modelling the endothelial cell niche crosstalk with endoderm, and reveals mechanisms by which endothelial cells promote hepatic specification of mouse ESC-derived endoderm cells through endothelial VEGFR2 activation and endoderm epigenetic modifications.

1. Introduction

The murine hepatic endoderm derives from the ventral foregut endoderm at E7.5 and specifies into hepatoblasts to form the liver bud at around E8.25 via BMP and FGF signaling provided by the adjacent septum transversum and cardiac mesoderm (Deutsch et al., 2001; Gordillo et al., 2015; Rossi et al., 2001). Growing evidence reported the key role of endothelial cells in triggering hepatic fate of the foregut endoderm. Indeed, liver buds do not develop in absence of functional endothelial cells in *Flk-1* null embryos (Matsumoto et al., 2001). In line with this study, we previously demonstrated that hepatic specification of mouse ESC-derived endoderm is controlled by endothelial cells through dual repression of Wnt and Notch pathways (Han et al., 2011). Although there are substantial evidence supporting the instructive role of endothelial cells for liver bud formation and specification, the mechanisms by which endothelial cells act are not fully understood.

During this last decade, numerous studies have provided compelling evidence that the development of multiple organs including the liver are controlled by epigenetic modifications by silencing or inducing organ specific genes. Epigenetic modifications include DNA methylation and histone alterations (Bernstein et al., 2007; Goldberg et al., 2007) such as methylation, acetylation, phosphorylation, ubiquitination, and sumoylation that play a critical role in chromatin architecture and hence gene transcription. Usually histone acetyltransferases (HATs)

Abbreviations: ESC, human embryonic stem cell; EB, embryoid body; AL End, mouse ESC-derived endoderm cells cultured alone; CC End, mouse ESC-derived endoderm cells co-cultured with D4T endothelial cells; FACS, fluorescence-activated cell sorting; AFP, alpha fetoprotein; ALB, albumin; TTR, transthyretin; VPA, valproic acid; 5-aza, 5-Aza-2′-deoxycytidine; GSK126, S-adenosyl-methionine-competitive small molecule inhibitor of EZH2 methyltransferase activity; DNMT, DNA methyltransferases; HMT, histone methyltransferases; HAT, histone acetyltransferase; HDAC, histone deacetyltransferases

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open the chromatin structure and activate gene expression, whereas hypoacetylation catalyzed by histone deacetyltransferases (HDACs) is correlated with reduced transcription or gene silencing (Sterner and Berger, 2000). DNA and histone methylation catalyzed by DNA methyltransferase (DNMTs) and Histone methyltransferase (HMTs) respectively are required for the recruitment of HDACs, therefore are mainly associated with gene repression (Vaissiere et al., 2008). Recently, few studies have associated specific epigenetic marks with liver development. Control of hepatic cell lineage differentiation by dynamic epigenetic histone modifications has been reported in mouse and human ESC cultures (Kim et al., 2011; Snykers et al., 2009; Vanhove et al., 2016). The in vivo evidence for the function of epigenetic marks in liver development results mostly from studies in zebrafish and mice. In zebrafish, knockout of *dnmt1* (Anderson et al., 2009), *dnmt2* (Rai et al., 2007), *dnmt3b* (Takayama et al., 2014) or the co-factor of DNMT1, *uhrf1,* (Mudbhary et al., 2014), leads to DNA hypomethylation and alters liver development, suggesting that DNMT activity is required for proper liver development. In the mouse, conditional knockout of the HMT *Enhancer of zeste homolog 2* (*Ezh2)* in Foxa3+ endoderm cells or reduction of the HAT P300 in *P300+/−* embryos significantly decrease the size of the liver bud at E9.5–10 accompanied with a diminution of hepatoblast numbers (Xu et al., 2011). Positive effect of Ezh2 on hepatoblast expansion was supported in vivo (Koike et al., 2014) and ex vivo following cell isolation and culture culture (Aoki et al., 2010). However the role of Ezh2 for hepatoblast differentiation into hepatocytes diverge depending on the in vivo knockout strategy (Koike et al., 2014) or ex vivo knockdown strategy (Aoki et al., 2010). It was indeed reported that EZh2 knockdown promotes hepatoblast differentiation into fetal hepatocytes by up-regulating transcription factors related to hepatocyte differentiation (Aoki et al., 2010).

Overall, liver specification is the result of a complex cross-talk between the foregut endoderm and the microenvironment to lead to endoderm gene network interaction that requires epigenetic modifications on multiple key factors and at specific times. In the present study, we dissected further the mechanisms by which endothelial cells trigger endoderm hepatic specification. Using our previously established in vitro mouse ESC system (Han et al., 2011), we compared transcriptome profiles of hepatic endoderm cells isolated from co-cultures and endoderm cells cultured alone, and uncovered that endothelial cells instruct liver specification of ESC-derived endoderm through endothelial VEGFR2 signaling and endoderm epigenetic modifications.

2. Materials and methods

2.1. ESC maintenance and differentiation

The mouse ESC line used is a double knock-in line with human CD4 targeted into the *Foxa2* locus and human CD25 into the *Foxa3* locus (Gadue et al., 2009). ESCs were cultured at 30,000 cells/ml to allow embryoid body (EB) formation in serum-free differentiation (SFD) media onto low-attachment petri dishes (Gouon-Evans et al., 2006). Day-2 EBs were dissociated, and 40,000 cells/ml cells were re-aggregated in SFD media supplemented with Activin-A (100 ng/ml). Day-5 EBs were dissociated with 0.25% trypsin/EDTA and endoderm cells (Foxa2+/Foxa3+) were purified by cell sorting and plated on matrigel-coated 48-well plates (80,000 cells/well) in the presence or the absence of D4T endothelial cells (4000 cells/well) in hepatic media for 3–8 days (Gouon-Evans et al., 2006). All cytokines except Activin-A (PeproTech) and bFGF (invitrogen) were purchased from R&D Systems.

2.2. Flow cytometry and cell sorting

Day-5 EBs were dissociated with 0.25% trypsin/EDTA. Endoderm cells were purified with a BD FACSAria II cell sorter using anti-hCD4-PE and anti-hCD25-APC antibodies and then cultured in hepatic media on matrigel-coated p48-well plates for 3 days. Day-8 differentiation cultures were dissociated and stained with anti-hCD4-PE and anti-CD31-APC antibodies followed by cell sorting. Flow cytometry analysis was done using FlowJo software (Tree Star Inc). Antibodies are listed in Supporting Information Table 1.

2.3. Deep RNA sequencing and data analysis

1μg of high quality total RNA was prepared from day-8 endoderm cells cultured alone or purified purified day-8 co-cultured endoderm cells using the Qiagen RNeasy Plus Micro kit. Each group had duplicates obtained from 2 separate differentiations. Deep RNA Sequencing was performed on the Illumina Genome Analyzer (HiSeq 2500) in the Genomics Core Facility at Icahn School of Medicine at Mount Sinai. A total of 24,373 transcripts of the whole mouse genome were analyzed. The number of reads for each transcript and reads per kilobase of a transcript per million mapped reads (RPKMs) were calculated and assigned to each transcript (Supplementary data-Deep RNA sequencing raw data). Duplicates were averaged and normalized to the initial expression level. Differential expression analysis was performed using DESeq and transcripts for further analysis were selected only if they also displayed at least a 2-fold change and the raw read count was > 100 in at least one sample. The genes whose expression changed between the co-cultured group and the alone group were hierarchically clustered with Cluster 3.0 and visualized using TreeView. Gene ontology analysis was performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov).

2.4. Immunostaining

CD31 immunostaining was performed on day-13 differentiated cells after fixation with 4% paraformaldehyde and blocking with the blocking buffer (Dako) by incubation with the CD31 antibody for 1 h at room temperature followed by incubation with the donkey anti-rat IgG-A488 secondary antibody. Cells were then permeabilized with 0.3% Triton X-100, blocked, and consecutively incubated with anti-AFP and anti-Foxa2 antibodies at 4 °C overnight, followed by the donkey anti-rabbit IgG-Cy3 and anti-goat-Cy5 secondary antibodies. Rat, rabbit or goat IgG (for CD31, AFP and Foxa2) were used in the negative control. The stained cells were finally counterstained with DAPI and visualized using a Leica fluorescent microscope and images captured using Leica software. Antibodies are listed in Supporting Information Table 1.

2.5. Western blotting

Day-13 differentiation cultures were harvested using 0.25% Trypsin/EDTA. Total protein lysates were obtained by RIPA buffer lysis supplemented with proteinase inhibitor cocktails. Total lysates were fractionated on a 4–12% gradient SDS-polyacrylamide gel and electroblotted on PVDF membranes. Chemiluminescence detection was performed according to manufacturer's instructions (Millipore). Antibodies are listed in Supporting Information Table 1.

2.6. Real-time qPCR

RNAs were reverse transcribed into cDNA using the Superscript III First-strand Synthesis System kit (Invitrogen). Quantitative Real Time-PCR (qPCR) was performed with a Roche System (LC480, Indianapolis, IN, http://www.roche.com). All experiments were done in triplicate using the Roche SYBR Green master mix. Primer sequences are listed in Supporting Information Table 2. Relative quantification was calculated using the comparative threshold (CT) cycle method and was normalized against the dCT of house-keeping gene *β-actin*.

2.7. Statistical analysis

Results are indicated as mean ± SD. For each group, samples from

3 or 6 individual experiments were analyzed, and different groups were compared using the *t*-test analysis. $p < .05$ was considered statistically significant; *, $p < .05$; **, $p < .01$; and ***, $p < .001$.

2.8. Key resources table

Those include the lists of antibodies (Table 1), primers (Table 2) and small molecules (Table 3).

3. Results

3.1. Transcript profiling analyses validate the endothelial cell-mediated hepatic specification of mouse ESC-derived endoderm cells

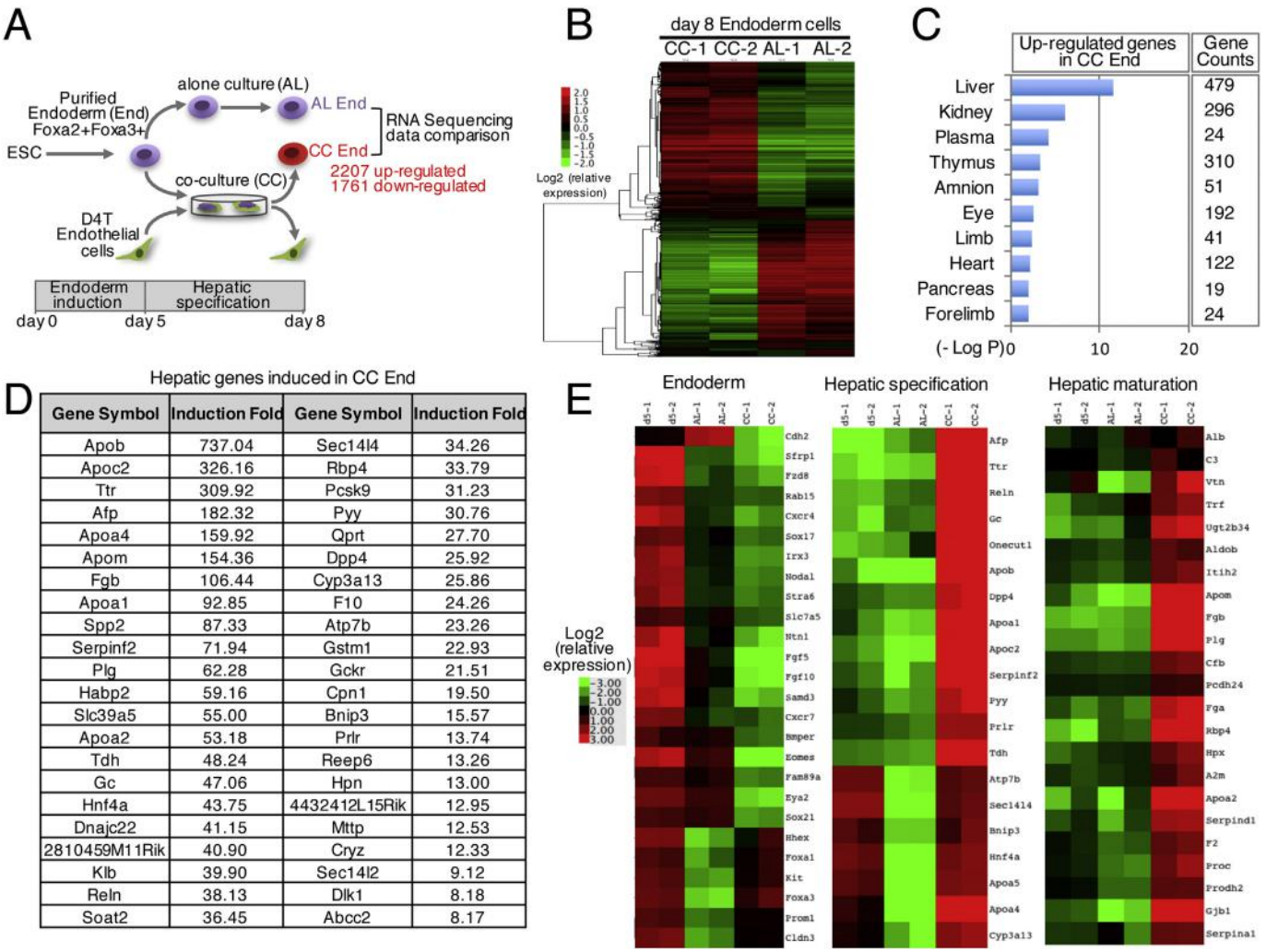
Our previous study demonstrated that hepatic specification and expansion of mouse ESC-derived endoderm is controlled by endothelial cells through dual repression of Wnt and Notch pathways in endoderm cells (Han et al., 2011). To further explore the mechanisms by which endothelial cells promote endoderm hepatic specification and cell proliferation, a mouse reporter ESC line in which hCD4 and hCD25 were targeted into the *Foxa2* and *Foxa3* loci respectively was used to faithfully track definitive endoderm cells (Gadue et al., 2009). Endoderm cells were generated in the presence of high doses of Activin-A and purified by cell sorting at day-5 of differentiation in the hCD4 + hCD25+ cell population representing the Foxa2 + Foxa3+ cells as previously described (Han et al., 2011). Purified endoderm cells were cultured for 3 days in hepatic medium either alone (alone endoderm cells: AL End) or in the presence of murine D4T endothelial cells (co-cultured endoderm cells: CC End). D4T endothelial cells were originally generated from day-4 mouse ESC differentiation cultures and further immortalized (Kennedy et al., 1997). Day-8 co-cultured endoderm and endothelial cells were dissociated and purified using hCD4 as a surrogate for the endoderm marker Foxa2 and the endothelial marker CD31 respectively (Fig. 1A). Our previous study indeed indicated that D4T cells express the endothelial marker CD31 and Flk-1/VEGFR2, while those markers were virtually absent in hCD4 + hCD25+ endoderm cells-derived hepatic cells (Han et al., 2011). Deep RNA sequencing was performed on purified day-8 AL End and CC End, and their transcript profiles compared. Out of 24,373 transcripts mapped, 2207 up-regulated genes (> 2-fold) and 1761 down-regulated genes (for at least 50%) were identified in CC End (Fig. 1B). Tissue specification analysis showed that among 2207 up-regulated genes, 479 were specifically restricted to liver (Fig. 1C), confirming our previous study demonstrating that co-culture of ESC-derived endoderm cells with endothelial cells specifically favors liver specification over other organ fate decision (Han et al., 2011). Examples of up-regulated hepatic genes were alphafeto protein (*Afp*) and transthyretin (*Ttr*), two early liver development markers (Gualdi et al., 1996; Makover et al., 1989) with 182.32- and 309.92-fold increase respectively. *HNF4α*, the master transcription factor regulating hepatic epithelium specification and fetal liver morphogenesis (Parviz et al., 2003), was induced 43.75-fold in co-cultures. Apolipoproteins (*Apob, Apoc2, Apoa4, Apom, Apoa1, Apoa2*), which are abundantly synthesized in hepatocytes, were highly induced in CC End with 53.18- to 737.04-fold increase (Fig. 1D). The heat maps (Fig. 1E) indicated that primitive streak endoderm markers such as *CXCR4, Sox17, Nodal, Eomes, Hhex, cKit* and *Foxa3* were highly enriched in day-5 purified endoderm cells, while markers for hepatic specification (*Afp,* gene encoding for the vitamin D binding protein *Gc, Ttr, Onecut-1, Apob*…) were strongly expressed in day-8 CC End. Genes related to initiation of hepatic maturation including *Apoa2, Rbp4, Fgb, Apom, Fga* were also upregulated in CC End. Although expression of some liver maturation markers (*Alb, Itih2, C3, Vtn* and *F2*) remained low in CC End, their transcript levels were higher than in AL End indicating that day-8 CC End recapitulates an early stage of hepatic differentiation as described in our previous study (Han et al., 2011). These high-throughput analyses supported our previous published study demonstrating that endothelial cell niche promotes mouse hepatic specification (Han et al., 2011) and validated the utility of the mouse ESC system to dissect further endothelial cell-mediated liver specification process.

3.2. Endothelial VEGFR2 activation indirectly mediates endoderm hepatic specification

Given that VEGF/VEGFR2 axis is a critical pathway in endothelial cell biology, we investigated the indirect impact of endothelial VEGFR2 activation for endoderm hepatic specification. VEGFA binding to VEGFR2 in endothelial cells activates several downstream signaling pathways including Ras/MAPK, ERK1/2/MAPK, FAK/paxillin, PI3K/AKT and Jak-STAT pathways that trigger multiple biological responses related to angiogenesis such as endothelial cell proliferation, survival, adhesion, and migration (Vieira et al., 2010). VEGFR2/Flk-1 is highly expressed in mouse D4T endothelial cells used in co-cultures, while it is absent in hepatic endoderm (Han et al., 2011). To investigate the indirect function of endothelial VEGFR2 activation on endoderm hepatic specification, inhibition assays of VEGFR2 were performed using the small molecule SU5416 (Fong et al., 1999), a potent and selective inhibitor of VEGFR2. Co-cultures of endoderm cells with D4T endothelial cells were treated with SU5416 from day-5 to day-13 in hepatic medium depleted of VEGF. Transcript levels of the four hepatic genes *Hnf4α*, *Afp*, *Ttr* and *Alb* were significantly reduced at day-13 in SU5416-treated co-cultures compared to those in control DMSO-treated co-cultures (Fig. 2A). Decreased levels of *Afp* transcripts in the presence of SU5416 was consistent with reduced levels of Afp proteins as assessed by immunostaining in the dish (Fig. 2B) and quantitatively by western blotting (Fig. 2C). Induction of Afp protein expression in CC End versus AL End was confirmed by immunostaining (Fig. 2B). Overall, these data indicate that activation of the VEGF/VEGFR2 axis in endothelial cells indirectly promotes endoderm hepatic specification.

3.3. Endoderm epigenetic modifications are associated with endothelial cell-mediated endoderm hepatic specification

To further explore the mechanisms by which endothelial cells drive endoderm hepatic specification and proliferation, we performed a gene ontology analysis of both sets of 2207 up-regulated genes and 1761 down-regulated genes in CC End. Ontology analysis showed that up-regulated genes were highly related to cell cycle and DNA replication including *Ccna2, Ccnb1, Ccnb2, Ccne1, Ccne2, Mki67, F2f1* and *Chek1* (Fig. 3A). Many global transcriptional regulation-related genes were up-regulated in CC End, such as *Tcfap4* and *Zfp* transcription factor family; as well as the master transcription factors for hepatic fate decision such as *Hhex*, hepatocyte nuclear factors (*Hnf4, Hnf1α, Hnf1β*) and Forkhead box proteins (*Foxa1, Foxa3, Foxm1, Foxo4*) (Fig. 3A), supporting again the key role of endothelial cells in liver specification. Function analyses indicated that a large fraction of up-regulated genes was significantly enriched for genes involved in lipid homeostasis, glucose metabolic process and hormone metabolism (Fig. 3A), which are the main functions of the liver. Interestingly, a significant number of up-regulated genes in CC End was related to epigenetic modifications including chromatin modification and DNA methylation such as DNA methyltransferases (*Dnmt1, Dnmt3b, Dnmt3a*), histone methyltransferases (HMTs such as *Ezh1* and *Suz12*), histone acetyltransferase (HAT1) and deacetyltransferases (HDACs: *Hdac10, Hdac5*, and *Sirt1*) (Fig. 3A). In contrast, the list of down-regulated genes was enriched for genes responsible for other organ commitment, such as brain, placenta, muscle and heart (Fig. 3B), validating the restricted role of D4T endothelial cells in endoderm hepatic fate decision. The predicted gene networks for up-regulated genes and down-regulated genes in CC End were performed using a cut-off of 4-fold or 25% (Fig. 3C,D). The up-regulated genes were highly associated with transcription factors critical for liver development (*HNF4α*), tissue growth (*Esr1*) or pluripotency (*Pou5f1*).



Gene Symbol	Induction Fold	Gene Symbol	Induction Fold
Apob	737.04	Sec14l4	34.26
Apoc2	326.16	Rbp4	33.79
Ttr	309.92	Pcsk9	31.23
Afp	182.32	Pyy	30.76
Apoa4	159.92	Qprt	27.70
Apom	154.36	Dpp4	25.92
Fgb	106.44	Cyp3a13	25.86
Apoa1	92.85	F10	24.26
Spp2	87.33	Atp7b	23.26
Serpinf2	71.94	Gstm1	22.93
Plg	62.28	Gckr	21.51
Habp2	59.16	Cpn1	19.50
Slc39a5	55.00	Bnip3	15.57
Apoa2	53.18	Prlr	13.74
Tdh	48.24	Reep6	13.26
Gc	47.06	Hpn	13.00
Hnf4a	43.75	4432412L15Rik	12.95
Dnajc22	41.15	Mttp	12.53
2810459M11Rik	40.90	Cryz	12.33
Klb	39.90	Sec14l2	9.12
Reln	38.13	Dlk1	8.18
Soat2	36.45	Abcc2	8.17

Fig. 1. Endothelial cell co-cultures promote endoderm hepatic specification.
(A) Illustration of the experimental design of endoderm cells harvested for deep-RNA sequencing. Day-5 Foxa2 + /Foxa3 + cells were cultured either alone (AL End) for 3 days or co-cultured with D4T cells for 3 days followed by cell purification from co-culture at day 8 (CC End). 2207 genes were up-regulated and 1761 genes were down-regulated in CC End compared to in AL End.
(B) Heat map summarizing relative transcript levels in CC End (CC) versus AL End (AL). Each group has 2 samples from 2 independent differentiations.
(C) Gene ontology analysis of up-regulated genes in CC End.
(D) List of the top 44 hepatic genes induced in CC End.
(E) Heat map in day-5 endoderm cells, day-8 CC End and day8- AL End comparing gene expression related to endoderm, hepatic specification and maturation.

Interestingly, many of the predicted genes were related to epigenetic modifications such as HDACs (*Hdac1, Hdac2, Hdac3, Hdac4* and the related complex *Sin3a*), and HMTs (*Ezh2* and the related complex *Suz12*) (Fig. 3C), supporting the tight relationship between epigenetic modifications and endothelial cell-mediated endoderm specification. Similar interactions were also reported between the down-regulated gene network (Fig. 3D), and HDACs (*Hdac1, Hdac2, Hdac3* and *Sin3a*), HMTs (*Ezh2, Suz12* and *Eed*), as well as DNMTs (*Dnmt1, Dnmt3a* and *Ndmt3b*) (Fig. 3D) supporting further the role of epigenetic modifications in endothelial cell-mediated endoderm hepatic specification.

3.4. DNA methylation, histone deacetylation and demethylation drive hepatic specification

To specifically explore the impact of DNA methylation, histone acetylation and methylation on endoderm hepatic specification co-cultured with D4T cells, small molecules modulating epigenetic modifications were used individually in co-cultures. Those included valproic acid (VPA), C646, 5-Aza-2′-deoxycytidine (5-aza), and GSK126. VPA is a global inhibitor for both class I and II HDACs by binding to the catalytic center of HDACs (Gottlicher et al., 2001). C646 is a reversible HAT inhibitor specific for p300/CBP, which competes with acetyl-CoA for the p300 Lys-CoA binding pocket and suppresses histone H3 and H4 acetylation (Bowers et al., 2010). 5-aza is extensively used as a DNMT inhibitor and leads to DNA demethylation. Finally, GSK126 is a highly selective small-molecule inhibiting HMT activity of Ezh2 and hence decreasing global H3K27me3 levels associated with gene silencing (McCabe et al., 2012). Concentrations of each small molecule were carefully optimized to prevent treatment-induced cell death that could have affected hepatic specification. Total cell numbers and percentages of CD31 + D4T cells by flow cytometry were determined for each treatment condition and subsequently endoderm cell numbers were extrapolated (Fig. 4A). Endoderm cell numbers were not significantly altered following VPA and GSK126 treatment that induce two opposite effects on hepatic specification as assessed by hepatic gene and protein expression (Fig. 4 B–H). Treatments with C646 and 5-Aza reduced endoderm cell numbers by 2-fold, although cell cultures remained healthy. Hepatic fate decision was examined at day-13 by evaluating transcript and protein levels of hepatic markers. Compared to DMSO, VPA completely abolished *Hnf4a, Afp*, *Ttr* and *Alb* transcript levels

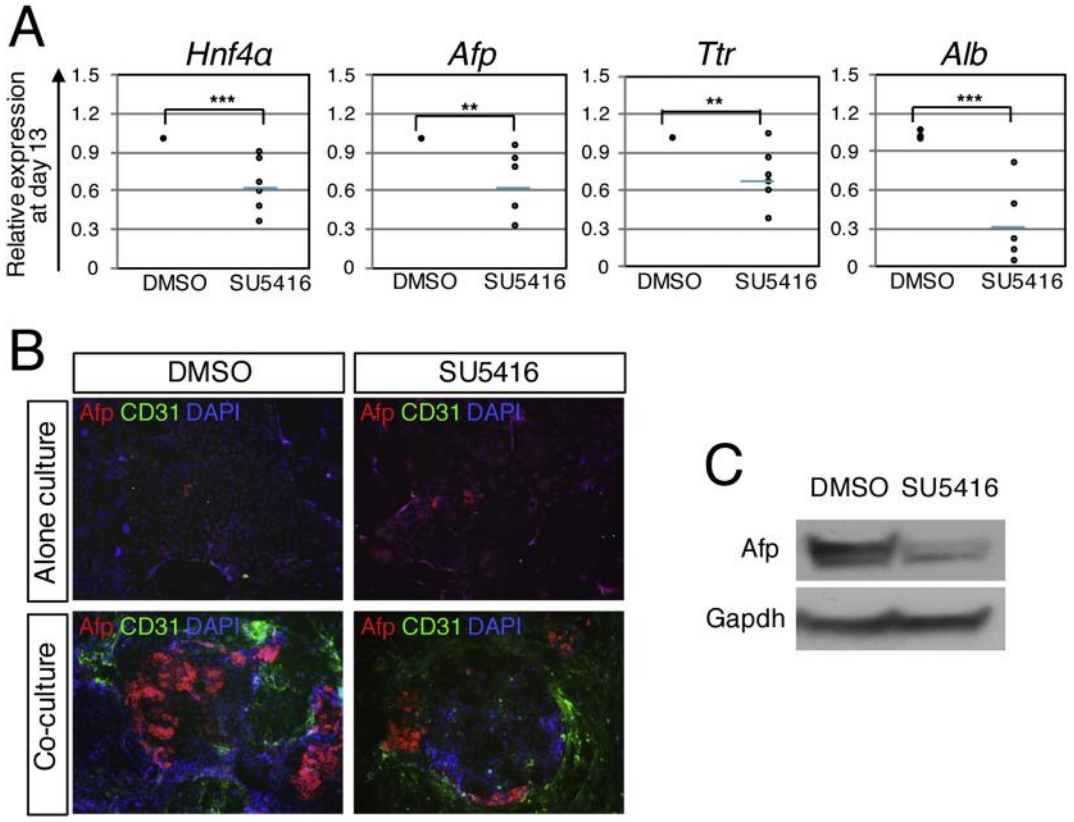


Fig. 2. Blocking VEGF signaling by SU5416 impedes endoderm hepatic specification.
(A) Relative transcript levels at day-13 of marker for hepatic endoderm (Hnf4α), hepatic specification (Afp and Ttr), and maturation (albumin, Alb) in endoderm cells cultured alone or co-cultured cells treated with DMSO or SU5416. n = 6 independent differentiations; *p < .05, **p < .01, ***p < .001.
(B) Immunostaining at day-13 for Afp (red) and CD31 (green) of endoderm cells cultured alone or co-cultured with D4T cells and treated with DMSO or SU5416.
(C) Western blot of Afp expression in day-13 co-cultured endoderm cells treated with DMSO or SU5416.

(Fig. 4B), indicating that histone deacetylation promotes hepatic specification. As a result, Afp proteins were undetectable in VPA-treated co-cultures as assessed by western blotting (Fig. 4C) and immunostaining (Fig. 4D: DMSO, 4E: VPA). C646 and 5-aza treatments significantly decreased transcript levels of *Hnf4α*, *Afp*, *Ttr* and *Alb* (Fig. 4B) as well as Afp protein levels (Fig. 4 C, F, G), although the effect was not as drastic as with VPA. However, blocking Ezh2 with GSK126 led to significant increase of *Hnf4α*, *Afp*, *Ttr* and *Alb* transcript levels (Fig. 4B) and augmentation of Afp protein (Fig. 4 C,H), supporting that histone methylation, most likely H3K27me3, is a barrier for hepatic fate decision. Altogether, our data indicate that endothelial cells indirectly mediate hepatic specification through epigenetic modifications in endoderm cells including histone acetylation, DNA methylation and histone demethylation.

4. Discussion

Understanding liver development is the foundation to efficiently generate functional hepatocytes from pluripotent stem cell (PSC) cultures. Although the time-line of liver development is well defined, the cross-talk between the prospective hepatic endoderm cells and adjacent cell niches remains elusive. The endothelial cell niche is recognized as a critical player in hepatic endoderm expansion to establish the murine liver bud (Matsumoto et al., 2001), as well as in hepatic differentiation of mouse ESC-derived endoderm mediated through repression of Wnt and Notch signaling (Han et al., 2011). This present study further uncovered that activation of VEGFR2 in D4T endothelial cells is required to promote hepatic cell fate of mouse ESC-derived endoderm, as it has recently been reported in human PSC-derived 3D liver buds (Camp et al., 2017). Further investigations are needed to identify the gaps that cross-link VEGFR2 activation in endothelial cells and hepatic fate decision of endoderm cells.

Comparison of the transcript profiling of CC End and AL End revealed that endoderm epigenetic modulations are triggered by the endothelial cell-mediated hepatic specification process. Modulations of epigenetic modifiers with small molecules provided a mechanism by which endothelial cells indirectly mediate hepatic specification in endoderm cells through epigenetic modifications. Given that off-target effects induced by small molecules cannot be fully ruled out, these findings will need to be confirmed using other means or additional small molecules to modulate epigenetic modifiers. Few studies using in vitro ESC system or in vivo and ex vivo mouse embryos have supported or complemented our findings. Interestingly, the timing of the use of small molecules affecting epigenetic marks altered variably hepatic cell fate. For instance, Sodium butyrate, a common HDAC inhibitor, was successfully used in ESC cultures to promote hepatic differentiation when included during endoderm induction (Duan et al., 2010; Hay et al., 2008). In our study, treatment of purified endoderm cells with the HDAC inhibitor, VPA, prevented them to differentiate into AFP+ hepatic cells, suggesting that HDACs exert different roles at different stages of liver development. Blocking the activity of the HAT P300 by C646 repressed endoderm hepatic gene transcript levels. Our finding was consistent with the observation of small liver buds in *P300*+/− embryos at E9.5 and decreased hepatic gene expression in purified hepatoblasts from these embryos (Xu et al., 2011). Similar to histone acetylation modifications, histone methylation status was also altered in endoderm cells undergoing specification in the presence of endothelial cells. Blocking the HMT Ezh2 by the specific inhibitor GSK126 led to higher expression of *Hnf4α, Afp, Ttr* and *Alb* transcripts and Afp proteins suggesting that histone demethylation promotes endoderm

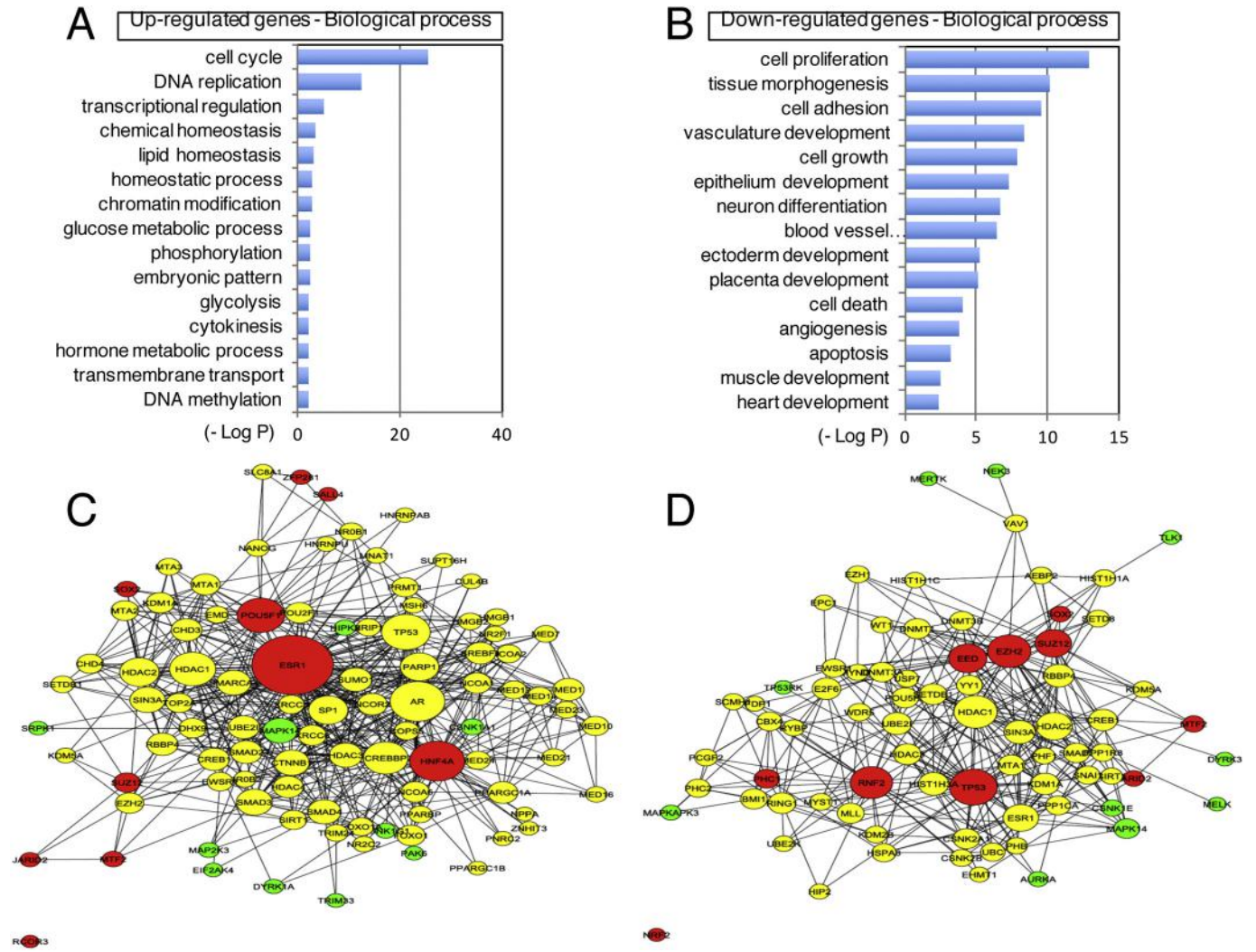


Fig. 3. Epigenetic modifications affect endoderm hepatic specification.
(A) Gene ontology (GO) of biological processes analysis on up-regulated genes in CC End cells.
(B) Gene ontology (GO) of biological processes analysis on down-regulated genes in CC End cells.
(C) Predicted gene network for up-regulated genes in CC End cells identifying HDACs and HMTs.
(D) Predicted gene network for down-regulated genes in CC End cells showing the connections of down-regulated genes with HDACs, HMTs and DNMTs.
(C, D) Transcription factors are shown in red; kinases in green and transcriptional co-factors in yellow. The size of the circles is correlated with the number of interactions with other candidate genes.

hepatic specification. The relationship between histone methylation and key master genes of liver development was recently studied by several groups. Ezh2 is highly expressed in hepatoblasts at E9.5 and its expression decreases during fetal liver outgrowth and maturation to be barely detected in E17.5, newborn and adult (Koike et al., 2014), suggesting that Ezh2 down-regulation is required for liver development. Similarly, HNF4α-deficient livers showed increased Ezh2 transcript levels associated with decreased levels of hepatic genes (Zhang et al., 2014), suggesting that maintenance of Ezh2 expression affects negatively liver maturation as revealed in our present study. However, conditional Ezh2 knockout in Foxa3 expressing cells results in small livers (Xu et al., 2011) suggesting that histone methylations are required for proper liver organogenesis. This conflict might be due to the complex outcome of Ezh2 knockout on multiple target genes and indirectly on multiple cell types within the liver. Interestingly, blocking DNA methylation by 5-aza decreased all hepatic gene transcript levels tested in this study. DNA methylation is mediated via DNMT and is usually associated with gene repression. Consequently, 5-aza-mediated DNA demethylation results in open chromatin structure, which allows accessibility of transcription factors to promoters and enhancers to activate gene expression (Christman, 2002). Here we proposed that the inhibitory roles of DNA demethylation on hepatic specification might act through inhibition of genes that repress hepatic fate decision. Consistent with our findings, Foxa2 gene expression has been reported to be strongly inhibited by 5-aza treatment or DNMT3b knockdown during endoderm development. Foxa2 gene displayed high DNA methylation in CXCR4+ (endoderm marker) tissues and low DNA methylation in CXCR4- tissues (Bahar Halpern et al., 2014). This study further supports our present finding indicating that HNF4α expression was repressed by 5-aza treatment.

5. Conclusion

In summary, our mouse ESC-based co-culture system offers an efficient platform to dissect the cross-talk between endothelial cells and endoderm cells upon early liver specification. These studies provide mechanisms by which endothelial cells promote hepatic specification of endoderm cells in a non-cell autonomous fashion through endothelial VEGFR2 activation and endoderm epigenetic modifications. Our findings provide new tools to optimize PSC hepatic specification protocols

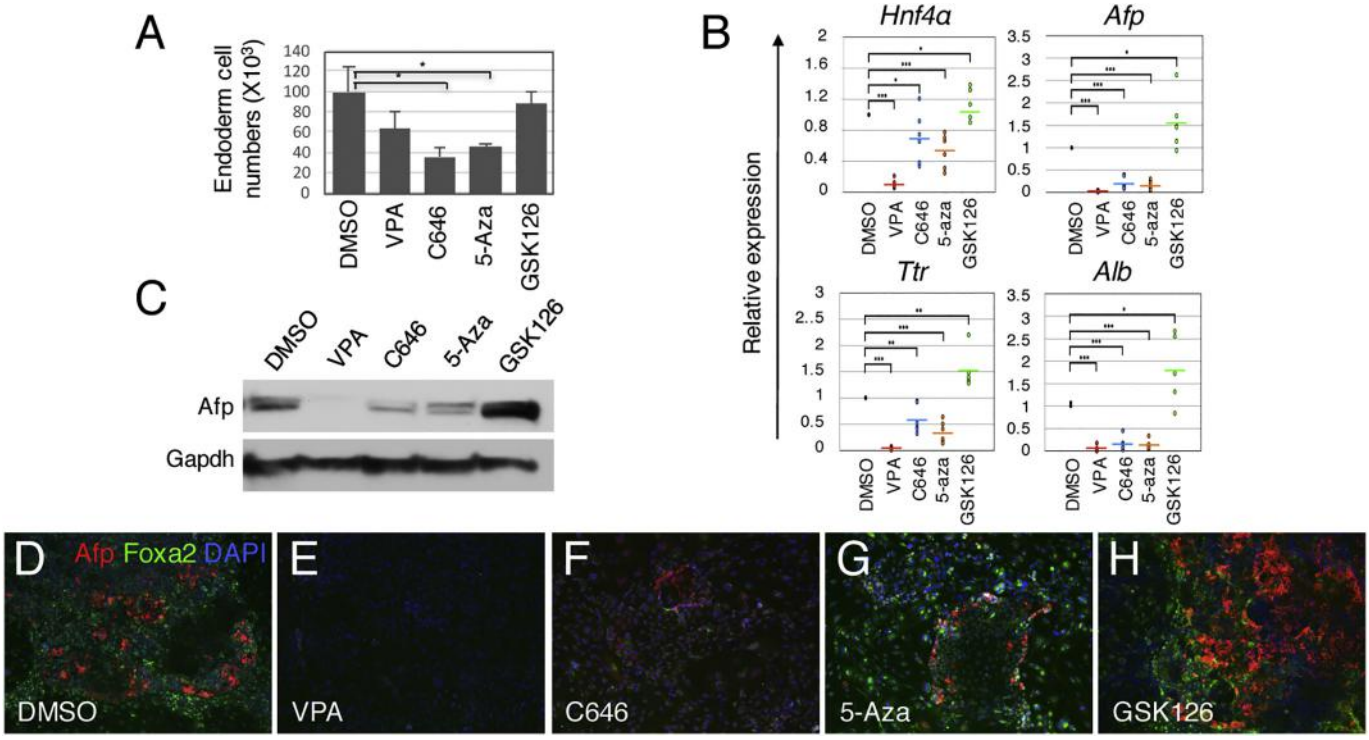


Fig. 4. Histone deacetylation, DNA methylation and histone demethylation induce endoderm hepatic specification.
(A) Endoderm cell numbers at day-13 co-cultured with D4T cells and treated with inhibitors for epigenetic modification. n = 3 independent differentiations; *p < .05, **p < .01, ***p < .001.
(B) Quantitative RT-PCR analysis at day-13 of genes related to hepatic endoderm (Hnf4α), hepatic specification (Afp and Ttr), and maturation (Alb) in endoderm cells co-cultured with D4T cells and treated with inhibitors for epigenetic modification. n = 6 independent differentiations; *p < .05, **p < .01, ***p < .001.
(C) Western blot of Afp expression in day-13 co-cultured endoderm cells treated with DMSO or epigenetic modification inhibitors.
(D–H) Immunostaining for Afp and Foxa2 of co-cultured endoderm cells at day-13 treated with DMSO or the inhibitors.

in order to generate functional hepatic cells for pre-clinical and clinical applications of PSC-based cell therapy for liver disease.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.06.004.

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