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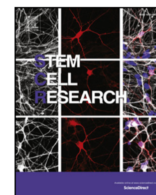

Endothelial cells instruct liver specification of embryonic stem cell-derived endoderm through endothelial VEGFR2 signaling and endoderm epigenetic modifications

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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

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