Liver Sinusoidal Endothelial Cells Suppress Bone Morphogenetic Protein 2 Production in Response to TGFβ Pathway Activation

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BACKGROUND AND AIMS: TGFβ/bone morphogenetic protein (BMP) signaling in the liver plays a critical role in liver disease. Growth factors, such as BMP2, BMP6, and TGFβ1, are released from LSECs and signal in a paracrine manner to hepatocytes and hepatic stellate cells to control systemic iron homeostasis and fibrotic processes, respectively. The misregulation of the TGFβ/BMP pathway affects expression of the iron-regulated hormone hepcidin, causing frequent iron overload and deficiency diseases. However, whether LSEC-secreted factors can act in an autocrine manner to maintain liver homeostasis has not been addressed so far.

APPROACH AND RESULTS: We analyzed publicly available RNA-sequencing data of mouse LSECs for ligand-receptor interactions and identified members of the TGFβ family (BMP2, BMP6, and TGFβ1) as ligands with the highest expression levels in LSECs that may signal in an autocrine manner. We next tested the soluble factors identified through in silico analysis in optimized murine LSEC primary cultures and mice. Exposure of murine LSEC primary cultures to these ligands shows that autocrine responses to BMP2 and BMP6 are blocked despite high expression levels of the required receptor complexes partially involving the inhibitor FK-506–binding protein 12. By contrast, LSECs respond efficiently to TGFβ1 treatment, which causes reduced expression of BMP2 through activation of activin receptor-like kinase 5.

CONCLUSIONS: These findings reveal that TGFβ1 signaling is functionally interlinked with BMP signaling in LSECs, suggesting druggable targets for the treatment of iron overload diseases associated with deficiency of the BMP2-regulated hormone hepcidin, such as hereditary hemochromatosis, β-thalassemia, and chronic liver diseases. (Hepatology 2021;74:2186-2200).

Transforming growth factor (TGF) β/bone morphogenetic protein (BMP) signaling is highly relevant for liver pathologies. TGFβ synthesis that occurs on liver injury is essential for the activation of hepatic stellate cells (HSCs) that play a critical role for the onset of the fibrotic process. Fibrosis is frequently hallmarked by iron overload and associated with low expression levels of the master regulator of iron homeostasis hepcidin. BMP2 and BMP6 control the expression of the hormone hepcidin in hepatocytes (HCs) through the BMP/mothers against decapentaplegic homolog (SMAD) signaling
pathway,\(^{(5,6)}\) and alterations of BMP-mediated hepcidin regulation cause disorders hallmarked by systemic and hepatic iron overload or deficiency.\(^{(7-9)}\) TGFβ superfamily ligands are sensed by a tetramer receptor composed of two type-II and two type-I receptors. The type-II receptors BMP receptor 2, activin receptor (ACVR) 2A, and ACVR2B phosphorylate the type-I receptors ACVR-like kinase (ALK) 2, ALK3, and ALK6 in the presence of BMP ligands. Subsequently, the type-I receptors phosphorylate the receptor-regulated SMAD (R-SMAD) 1, 5, and 8, which translocate into the nuclei together with the common SMAD4 (co-SMAD) and induce the expression of target genes hallmarked by BMP responsive elements. TGFβ ligands bind the type-II receptors TGFβ receptor 2, ACVR2A, and ACVR2B and the type-I receptors ALK1 and ALK5. SMAD2/3 signaling is the canonical pathway activated by TGFβ-ALK5, which induces the expression of genes containing SMAD responsive elements. Moreover, TGFβ ligands can also activate SMAD1/5/8 signaling through ALK1 and ALK5 type-I receptors. Both signaling pathways induce inhibitory SMAD 6 and 7, which then block the phosphorylation or the translocation of the R-SMADs (Fig. 1C).\(^{(10-12)}\)

Nonparenchymal cells (NPCs) of the liver express the highest levels of TGFβ/BMP ligands.\(^{(13-15)}\) In particular, liver sinusoidal endothelial cells (LSECs) play a central role in liver organ communication as they control the TGFβ-mediated activation of HSCs\(^{(16,17)}\) as well as regulation of hepcidin in HCs through the secretion of BMP2 and BMP6 ligands.\(^{(18)}\) LSECs are a subtype of specialized endothelial cells characterized by absence of basal lamina and presence of fenestrae.\(^{(17)}\) LSECs account for approximately 15% of the total number of liver cells and only 3% of the total liver volume.\(^{(19,20)}\) Experimentation with primary mouse LSECs is challenging due to the difficulties of isolating a sufficient number of LSECs with high purity.\(^{(17)}\) Due to their specialized phenotype, cell lines that have been generated thus far do not accurately reflect on primary LSECs. Therefore, there is a demand for the optimization of protocols that provide primary murine LSECs that can serve as a suitable model for biological studies. LSECs form the liver sinusoids and provide a hepatic reservoir of growth factors, such as TGFβ/BMPs. Several studies have focused on the paracrine role of growth factors released by LSECs. However, information on whether they engage in autocrine signaling in the liver is currently lacking.

Here, we studied the autocrine effects of growth factors produced by LSECs in a highly pure and undifferentiated preparation of primary LSECs. We demonstrate that LSECs maintain intrinsic mechanisms, rendering them resistant to BMP2 and BMP6 stimulation. By contrast, they efficiently respond to the profibrogenic cytokine TGFβ1, which decreases the hepcidin regulator Bmp2 through ALK5 activation, possibly providing insight into molecular mechanisms involved in BMP-controlled hepcidin regulation in various liver diseases.

**Material and Methods**

### ANIMAL EXPERIMENTATION

Mice and rats were housed in the specific-pathogen free (SPF) barrier at the Interfakultäre Biomedizinische Forschungseinrichtung (IBF) animal facility at the University of Heidelberg (Germany), at the animal facility of the Medical Faculty Mannheim, Heidelberg.
University (Germany) or at EMBL's Laboratory Animal Resources (LAR). Animals were provided a constant light-dark cycle and maintained on a standard diet containing 200 ppm iron with *ad libitum* access to food and water. All mouse breeding and animal experiments were approved by and conducted following the guidelines of Regierungspräsidium Karlsruhe (T60/19, T84/18, T75/17, G172/15, G216/16, T70-17, T42-20) and EMBL Institutional Animal Care and Use Committee.
FIG. 1. Identification of ligand–receptor interactions in LSECs. TPM of genes expressed in LSECs isolated from four wild-type mice have been calculated based on RNA-sequencing data deposited in the GEO database (GSE135790). (A) Ligand-receptor interaction analysis. The circular plot shows the direct interaction between ligands (light gray) and their receptors (dark gray) through connecting links. The colors of the links represent the sum of ligand and receptor expression in TPM. (B) Pathway enrichment analysis was performed by Enrichr. Enriched pathways are organized based on the combined score of P value and odd ratio. (C) Schematic representation of the BMP/TGFβ signaling pathways. mRNA expression of (D) BMP and TGFβ ligands, (E) type-II receptors, (F) type-I receptors, and (G) SMADs (receptor-regulated SMAD, common-SMAD) are represented in TPM. Abbreviations: Acvr1, Activin receptor-like kinase 1; Adm, adenomedullin; AGE-RAGE, advanced glycation endproducts-receptor for advanced glycation endproducts; Akt, protein kinase B; Angpt, angiopoietin; ApoE, apolipoprotein E; BMPRE, BMP responsive element; Calcr, calcitonin receptor like receptor; Calr, calreticulin; Col4a1, collagen type IV alpha 1 chain; Efna1, ephrin A1; Flt4, fms related receptor tyrosine kinase 4; Gadd, growth arrest specific 6; Gpr182, G protein-coupled receptor 182; Inhbb, inhibin subunit beta B; Igf1, insulin like growth factor 1; Itgb, integrin subunit alpha; Itga, integrin subunit alpha; Kdr, kinase insert domain receptor; Lamb2, laminin subunit beta 2; Ldlr, low density lipoprotein receptor; MAPK, mitogen-activated protein kinase; Mertk, MER proto-oncogene, tyrosine kinase; Nrpn, neuropilin; Ntn, netrin; PI3K, phosphoinositide 3-kinase; Ramp, receptor activity modifying protein; Rap, Ras-related protein; Ras, rat sarcoma; Rpsa, ribosomal protein SA; SBE, SMAD responsive element; Scarf, scavenger receptor class F member 1; Sema3f, semaphorin 3F; Tek, angiopoietin-1 receptor; Tgfb1, TGFβ receptor; TPM, transcripts per million; Unc5a, unc-5 netrin receptor A.

PREPARATION OF HCs AND LSECs FOR PRIMARY CULTURES

Liver perfusion was performed following a standard two-step perfusion method. The liver of C57BL/6N wild-type mice (8-10 weeks old for HC preparation; 13-16 weeks old for LSEC preparation) or Wistar rats (7-9 weeks old) was perfused with liver perfusion (20 mL for mice; 100 mL for rats) (Life Technologies, #17701038) and liver digest medium (20 mL for mice; 100 mL for rats) (Life Technologies, #17703-034) through the vena cava inferior in order to remove infiltrating blood and dissociate the extracellular matrix, respectively. Cava vein was cannulated with a 27G x 3/4” needle with perfusion speed of 5 mL/min. The liver capsule was mechanically disrupted and passed through 100 μm and 70 μm strainers to obtain a single cell suspension of liver cells resuspended in HC wash medium (Life Technologies, #17704-024). Liver cell suspension was centrifuged for 5 minutes at 50g (4°C) to separate HCs and nonparenchymal cells (NPCs). HCs were plated into collagen-coated plates (Life Technologies, #A1048301) in a concentration of 250,000 cells/mL. For the preparation of LSECs, protocols described in Zhang et al., Cheluvappa, and Hansen et al. have been followed with modifications. After the two-step perfusion described, NPCs were separated through density gradient isolation with OptiPrep (Sigma Aldrich, #D1556): (a) NPCs were centrifuged at 400g for 10 minutes at 4°C; (b) the pellet of NPCs was resuspended in 5 mL of OptiPrep 17.6%, then 5 mL of OptiPrep 8.2% and 2 mL of B-PBS (PBS + 0.1% BSA) were carefully loaded on top of cell suspension; (c) OptiPrep gradient was centrifuged at 1,400g for 17 minutes without brake at room temperature (RT). This step allows the isolation of a mixed suspension of LSECs and Kupffer cells (KCs) (cell fraction between the interface of OptiPrep 8.2% and 17.6%). Contrary to LSECs, KCs adhere rapidly to the cell culture dishes. The adherence step allows for the separation between LSECs and KCs. Thus, LSECs/KCs were resuspended in LSEC medium (see section below) and incubated at 37°C for 15 minutes (step repeated 2 times). Nonadherent cells (LSECs) were recovered and plated in collagen-coated plates at a concentration of 2 x 10^6 cells/mL and maintained in 20% oxygen tension. When HCs and LSECs were cultured together, half of the cell number indicated above was used. LSECs and hepatocytes were used within 30 hours after plating.

PREPARATION OF HCs AND LSECs FOR RNA EXTRACTION

HCs were isolated from C57BL/6N mice by using the protocol described above and immediately snap-frozen to preserve high RNA quality. LSECs have been isolated from C57BL/6N mice by using the protocol described with minor modifications. Briefly, after the density gradient isolation, KCs were depleted by using F4/80 antibody conjugated with magnetic microbeads (Miltenyi Biotec, #130-110-443) following the manufacturer’s protocol. Then, LSECs were
immunoselected by using the LSECs specific marker CD146 (Miltenyi Biotecm, #130-092-007).

**CELL CULTURE AND TREATMENTS**

Primary HCs were cultured in William's E medium (Life Technologies, #32551-020) supplemented with 4% fetal bovine serum (FBS) and 1% penicillin/streptomycin. LSECs were maintained in William's E medium supplemented with 4% FBS, 1% penicillin/streptomycin, and 10 ng/mL VEGF (PeproTech, #450-32). Both primary HCs and LSECs were serum starved in William's E medium supplemented with 10 ng/mL VEGF for 2 hours and then treated with 50 ng/mL BMP2 (PeproTech, #120-02C) or 50 ng/mL BMP6 (PeproTech, #120-06) or 5 ng/mL TGFβ1 (PeproTech, #100-21) for 4 or 15 hours. When indicated, 1 μg/mL tacrolimus (FK-506; provided by Dr. Laura Silvestri, San Raffaele Hospital) or 5 μM SB431542 or 500 nM K02288 were added. Bone marrow–derived macrophages (BMDMs) and HSCs were serum starved for 2 hours in the respective mediums and treated for 4 hours with 5 ng/mL TGFβ1.

**SCANNING ELECTRON MICROSCOPY**

LSECs were seeded on 1.2 cm cover slips coated with rat tail collagen I (Life Technologies, #A1048301) and chemically fixed after 6, 20, and 30 hours with 2.5% glutaraldehyde + 4% paraformaldehyde + 4% sucrose in 0.1 M PHEM buffer pH 7.2. Fixed cells were then successively treated with 1% osmium tetroxide/0.8% K4Fe(CN)6 (2 hours, 4°C), 1% tannic acid (1 hour, RT), and 1% uranyl acetate (1 hour, RT). Dehydration followed with graded ethanol and infusion with graded hexamethyldisilazane. Coverslips were then mounted on a scanning electron microscopy (SEM) stub with a carbon tab. Imaging were performed at 5 kV, 0.40 μA, and 700 pa.

**STATISTICAL ANALYSES**

Statistical analyses were performed using Prism v7 (GraphPad Software, San Diego, CA). Data are shown as mean ± SEM, and two-tailed Student t test was calculated when shown. P values < 0.05 (*), <0.01 (**), <0.001 (***) and <0.0001 (****) are indicated.

**Results**

**LSECs EXPRESS A DEFINED PATTERN OF TGFβ SUPERFAMILY LIGANDS AND RECEPTORS**

LSECs secrete growth factors, such as TGFβ family ligands, that act in a paracrine manner in liver-associated diseases, like hemochromatosis and fibrosis. We analyzed publicly available bulk RNA-sequencing data of LSECs isolated from wild-type mice (gene expression omnibus [GEO]: GSE135790) and applied ligand-receptor interaction analysis to predict possible signaling pathways that can be activated in an autocrine manner. Ligand–receptor interaction analysis implies that the higher the mRNA expression levels of ligands and receptors are, the greater the probability is that the signaling pathway is active. We selected ligand-receptor interactions in which the ligand is a secreted factor and focused on interactions already described in literature (Supporting Table S1). Following these criteria, 30 candidates appear to be relevant in LSECs (Fig. 1A, Supporting Table S1). Pathway enrichment analysis of genes identified by the ligand-receptor interaction analysis revealed members of the TGFβ superfamily as overrepresented (Fig. 1B, Supporting Fig. S1, Supporting Table S2). We hypothesized that BMP/TGFβ ligands released by LSECs may play a relevant role, not only as paracrine regulators but also as autocrine factors mediating LSECs.

Figure 1C shows a simplified scheme of players involved in BMP/TGFβ signaling pathway. To better understand which ligands and signaling components may be active in LSECs and to validate the RNA-sequencing results, we compared the mRNA expression of all BMP/TGFβ ligands (Fig. 1D) and their relative type-II (Fig. 1E) and type-I (Fig. 1F) receptors. As shown in Fig. 1D, BMP2, BMP6, and TGFβ1 are the most highly expressed ligands. Type-II receptors are all expressed (Fig. 1E), whereas only the type-I receptors ALK2, ALK1, and ALK5 are detectable in LSECs (Fig. 1F). In addition, genes involved in the transmission of signaling (R-SMAD and co-SMAD) are expressed (Fig. 1G).

Based on these results obtained by bioinformatics analysis, we hypothesized that several factors secreted by LSECs can act in an autocrine manner. Among
these, BMP2, BMP6, and TGFβ1 were the most promising candidates and were selected for further investigation.

HIGHLY PURE PREPARATION OF PRIMARY MURINE LSECs

To investigate possible autocrine effects of BMP2, BMP6, and TGFβ1 in LSECs, we optimized a protocol for the preparation of primary murine LSECs and carefully assessed purity and quality of the isolated cell preparation. Following liver perfusion, the NPCs fraction was separated based on cell density. Next, an adherence step separated LSECs from KCs (resident macrophages) (Fig. 2A). Approximately 3 million and 70 million LSECs with a viability of 99% can be isolated per mouse and rat, respectively. Isolated cells grew in a monolayer, loosely apposed, whereby the shape of the cells resembled a “cobblestone” pattern. Each cell showed an elongated or polygonal shape with a boat-shaped or irregular appearance (Fig. 2B, Supporting Fig. S2A), similar to previous descriptions of endothelial cells in culture. LSECs can undergo a transdifferentiation process, which is characterized by a progressive loss of fenestrae. Therefore, we performed SEM at 6, 20, and 30 hours after plating to evaluate the quality of the cell preparation and thus cell fenestration over time in mouse LSECs. Fenestrae are grouped in sieve plates (Fig. 2C), and they are mostly prevalent in cell islands (data not shown), suggesting that density of cell plating is a determinant for having a high-quality preparation of primary LSECs. Furthermore, fenestrae are detected in cells cultivated for an extended period of time (Fig. 2C; 30 hours), confirming the high quality of the primary cell preparation. We then analyzed the purity of mouse LSECs by FACS, assessing the expression of LSEC-specific surface markers CD146 and stabulin 2 (STAB2). LSECs displayed 94% viability, as determined by the percentage of 7AAD positive cells (Fig. 2D). Less than 1% of cells are single positive for CD146 or STAB2, and ≈95% cells are double positive for CD146 or STAB2 (Fig. 2E, Supporting Fig. S2B). To exclude the presence of KCs, we analyzed the surface expression of the macrophage marker F4/80. The percentage of F4/80 single positive cells is near 0%, yet we identified a small fraction (≈4%) expressing both macrophage and LSEC markers (Fig. 2F,G, Supporting Fig. S2C). Similarly, rat LSEC viability is ≈99% (Supporting Fig. S2D) and purity = 94%, as shown by FACS analysis of the LSEC marker SE-1 and the macrophage marker CD163 (Supporting Fig. S2E,F). Thus, our protocol establishes a preparation of pure primary mouse and rat LSECs without applying antibodies for cell selection.

PRIMARY MURINE LSECs DO NOT RESPOND TO BMP2 AND BMP6

We first tested whether primary LSEC cultures isolated from mouse and rat activate the SMAD1/5/8 signaling pathway in response to a short (4 hours) treatment with BMP2 or BMP6. As a control, primary HCs were subjected to identical treatment conditions. As expected, mRNA expression of the canonical BMP-SMAD target genes inhibitor of differentiation 1 (Id1), Smad6, Smad7, and atonal BHLH transcription factor 8 (Atoh8) were induced in HCs on BMP2 and BMP6 treatment (Fig. 3A,B, Supporting Fig. S3A). Surprisingly, SMAD signaling was not activated in primary LSECs isolated from mice (Fig. 3C,D) and rats (Supporting Fig. S3B). We next explored whether a longer treatment would be required for LSECs to respond to BMP2 and BMP6 ligands. Similar to the 4-hour time point, none of the SMAD1/5/8 target genes (Id1, Smad6, Smad7, and Atoh8) were induced after 15 hours (Supporting Fig. S3F,G). Again, primary mouse HCs, treated in parallel, showed the expected increase in mRNA expression of Id1, Smad6, Smad7, and Atoh8 (Supporting Fig. S3C,D). To further explore this finding, we incubated primary LSECs and HCs with the ATP-mimetic drug dorsomorphin, an inhibitor of all BMP receptors. Independent of the cell type, we observed a reduction of BMP-SMAD target genes (Supporting Fig. S3E,H), suggesting that BMP receptors in LSECs are, in principle, active. Comparable results were obtained in primary cultured LSECs and HCs from rats (Supporting Fig. S3A,B).

To understand why LSECs did not respond to BMP treatment, we analyzed mRNA expression of BMP-SMAD pathway inhibitors in the RNA-sequencing data of LSECs and HCs (GEO: GSE135790). Interestingly, inhibitors of ligands, receptors and signaling transducers, are expressed to higher levels in LSECs compared with HCs (Supporting Fig. S3I), suggesting that the response to BMP ligands may be attenuated by several
inhibitory mechanisms. The immunophilin FK-506–binding protein 1A (Fkbp1a; FK-506–binding protein 12 [FKBP12]) was selected as a promising candidate for further functional validation, as it is the most highly expressed BMP-SMAD inhibitor (Supporting Fig. S3I). It binds to the glycine-serine–rich domain of BMP type-I receptors maintaining them in an inactive conformation. Data have
demonstrated that FKBP12 binding to ALK2 can be inhibited by FK-506, inducing an uncontrolled activation of SMAD1/5/8 signaling. ALK2 is the BMP type-I receptor mainly expressed in LSECs (Fig. 1F) and is preferentially activated by the BMP6 ligand. We found that FKBP12 (Fkbp1a) is expressed in a ratio 18:1 compared with ALK2 (Acvr1) in LSECs (Supporting Fig. S3J). Therefore, we hypothesized that FKBP12 may inhibit the BMP-mediated activation of SMAD1/5/8 signaling in LSECs. To address this further, we treated mouse LSECs with the ALK2 ligand BMP6 with and without FK-506, allowing us to study responses of BMP6 when ALK2 is not inhibited by FKBP12. We observed that FK-506 alone induces the activation of ALK2, as indicated by Id1 (Fig. 3E), Smad6 (Fig. 3F), and Smad7 (Fig. 3G) up-regulation, whereby the fold-induction of SMAD target genes is more pronounced when FK-506 and BMP6 treatments are combined (Fig. 3E-G).

Taken together, our data show that canonical SMAD target genes are not induced in LSECs incubated with BMP2 and BMP6. This suggests that LSECs have intrinsic inhibitory mechanisms that block autocrine regulation by BMP2 and BMP6. Repression of the ALK2-inhibitor FKBP12 by FK-506 shows less-than-additive effects with BMP6, suggesting that it only partially contributes to the inhibition of the BMP2 and BMP6 responses.

**LSECs DO NOT RESPOND TO THE INCREASED BMP6 PRODUCTION IN MOUSE MODELS OF IRON OVERLOAD**

Recent studies showed that LSECs release BMP2 and BMP6 to induce hepcidin expression in HCs, which in turn controls dietary iron absorption and iron distribution in the body. BMP6 is strongly induced in mice with systemic iron overload. To assess whether the unresponsiveness of LSECs to BMP6 holds true in mouse models of iron overload, we studied ferroportin (Fpn) C326S mice (solute carrier family 40 member 1 [Slc40a1]C326S/C326S), which ubiquitously express a hepcidin-resistant mutant of the iron exporter Fpn, causing severe iron accumulation in several tissues such as blood, liver, and pancreas. Among the LSEC-expressed ligands BMP2, BMP6, and TGFβ1, only BMP6 expression is induced (Supporting Fig. S4A and data not shown). In parallel, the BMP-SMAD target genes Id1, Smad7, and Atoh8 (Supporting Fig. S4B-D) are increased in the total liver of Fpn(C326S) mice. We next isolated HCs and LSECs from Fpn(C326S) mice and analyzed the mRNA expression of BMP6 target genes. As shown in Fig. 4, the expression of Id1, Smad6, and Atoh8 is increased in HCs isolated from Fpn(C326S) mice (Fig. 4A), whereas their expression is unchanged in LSECs (Fig. 4B). To exclude that alterations in cellular iron distribution in Fpn(C326S) mice may cause unresponsiveness of LSECs to BMP6, we analyzed wild-type mice with systemic iron overload due to an iron-loaded diet. Mice were given an iron-loaded diet for 4 weeks, a sufficient time span to develop systemic and hepatic iron overload, as reported. As expected, mRNA expression of Bmp6 (Supporting Fig. S4E) and its downstream target genes are increased in total RNA preparations of liver and primary HCs (Supporting Fig. S4F-H, Fig. 4C). By contrast, the mRNA levels of BMP6-target genes in primary LSECs isolated from iron-loaded and control mice are comparable (Fig. 4D). These results confirm that LSECs are unable to respond to increased levels of BMP6 in vivo.
FIG. 3. Mouse LSECs are resistant to autocrine control by BMP2 and BMP6. Primary mouse HCs and LSECs were incubated with (A,C) 50 ng/mL of BMP2 and (B,D) 50 ng/mL of BMP6 for 4 hours in serum-free medium. (A-D) The mRNA expression of Id1, Smad6, Smad7, and Atoh8 was analyzed by qRT-PCR. Data are represented as fold-change compared with vehicle-treated cells. (E-G) Primary mouse LSECs were pretreated with 1 μg/mL of FK-506 (4 hours), then 50 ng/mL of BMP6 were added for 4 hours. (E) Id1, (F) Smad6, and (G) Smad7 mRNA expression was analyzed by qRT-PCR. Biological replicates of representative experiments are shown. mRNA expression data were normalized to the housekeeping gene Rpl19. Data are reported as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction; Rpl19, ribosomal protein L19; ut, untreated.
We showed that LSECs express TGFβ1 as well as receptors and signaling molecules to respond to TGFβ1 ligands (Fig. 1). We next analyzed the sensitivity of LSECs for TGFβ1. Primary mouse and rat LSECs were treated with TGFβ1 for 4 hours (Fig. 5A-C, Supporting Fig. S5). The expression of the SMAD2/3 target gene serpin family E member 1 (Pai1) increases on TGFβ1 treatment (Fig. 5A, Supporting Fig. S5A), demonstrating that, in contrast to BMP2 and BMP6, LSECs respond to increased levels of TGFβ1. Studies in several cell types and tissues indicate that TGFβ1 signaling is interlinked with the BMP pathway at the posttranscriptional and/or transcriptional levels. (36-39) Thus, we analyzed Bmp2 and Bmp6 expression in TGFβ1-treated LSECs. Interestingly, Bmp2 (Fig. 5B, Supporting Fig. S5B) is strongly down-regulated by TGFβ1 treatment, whereas Bmp6 expression is not affected (Fig. 5C, Supporting Fig. S5C), both in mouse and rat primary LSECs. Therefore these data demonstrate that TGFβ1 can dramatically regulate the growth factor BMP2 in LSECs.
FIG. 5. TGFβ1 is a repressor of Bmp2. (A-C) Mouse LSECs were treated with 5 ng/mL of TGFβ1 for 4 hours in serum-free medium. Total RNA was extracted and retrotranscribed and (A) Pai1, (B) Bmp2, and (C) Bmp6 mRNA expression were determined by qRT-PCR. Rpl19 was used as a housekeeping gene. Data from one representative experiment of three independent experiments are shown. (D,E) Twelve-week-old male wild-type mice were intravenously injected with TGFβ1 (0.1 μg/g of mouse) or vehicle and sacrificed 2 hours later. (D) Activity of the SMAD1/5/8 or SMAD2/3 signaling pathway was assessed by detecting phospho-SMAD5 and phospho-SMAD3 by western blot. Total SMAD5, total SMAD3, and β-actin were used as loading controls. (E) RNA was extracted from total liver, and Bmp2 mRNA expression was analyzed by qRT-PCR and normalized to the housekeeping gene Rpl19. (F,G) Primary mouse LSECs were incubated for 3 hours with 5 μM SB431542, and then 5 ng/mL of TGFβ1 was added for 4 hours (0% FBS). (H,I) Primary mouse LSECs were transfected with 30 nM of small interfering RNA (siRNA) targeting ALK5 or control for 44 hours, and 5 ng/mL TGFβ1 was added for an additional 4 hours. mRNA expression of (F,H) Pai1 and (G,I) Bmp2 were analyzed and normalized to the housekeeping gene Hprt1. Three to five biological replicates are shown. Data are reported as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Abbreviations: ctrl, control; Hprt, hypoxanthine-guanine phosphoribosyltransferase; MW, molecular weight; Pai1, serpin family E member 1; qRT-PCR, quantitative real-time polymerase chain reaction; Rpl19, ribosomal protein L19.
LSECs are the major source of BMP2 in the liver, but other cell types, such as resident macrophages (KCs), HSCs, or HCs, express Bmp2, albeit to a much lower level (Supporting Fig. S6A). We next tested whether TGFβ1 also decreases mRNA expression of Bmp2 in other liver cell types. We show that primary HSCs (Supporting Fig. S6C), primary HCs (Supporting Fig. S6D), and BMDMs (a surrogate for KCs) (Supporting Fig. S6B) display repressed Bmp2 mRNA expression on TGFβ1 treatment. Of note, LSECs and macrophages exert a more pronounced response to TGFβ1, as indicated by the activation of the TGFβ1-target genes Pai1 and Smad7, and a higher fold-reduction of Bmp2.

In order to validate the TGFβ1-dependent Bmp2 inhibition in vivo, we injected TGFβ1 in wild-type mice. As expected, TGFβ1 induces activation of both the SMAD1/5/8 and SMAD2/3 signaling pathways (Fig. 5D). The acute injection of TGFβ1 significantly reduced Bmp2 mRNA expression (Fig. 5E), thus confirming our data in primary cells.

**TGFβ1-DEPENDENT Bmp2 REGULATION IS CONTROLLED BY ALK5 RECEPTOR ACTIVITY**

TGFβ1 signals through the type-I receptors ALK1 or ALK5 (Fig. 1C). In order to identify the ligand-receptor interaction that induces Bmp2 down-regulation, we incubated primary mouse LSECs with TGFβ1 in the presence or absence of the ALK1 or ALK5 inhibitors K02288 or SB431542, respectively. Treatment with the ALK1 inhibitor did not affect the induction of TGFβ1 target genes or Bmp2 suppression (Supporting Fig. S7A-C). By contrast, the incubation of LSECs with the ALK5 inhibitor strongly attenuates the induction of the TGFβ1 target genes Pai1 and Smad7 (Fig. 5F, Supporting Fig. S7E). Importantly, the inhibition of ALK5 activity under basal conditions increases the expression of Bmp2 and completely abolishes the TGFβ1-mediated Bmp2 reduction (Fig. 5G, Supporting Fig. S5B). Bmp6 expression is not affected by any of these treatments (Supporting Fig. S5C, S7D,F). These data were validated in LSECs subjected to RNAi-mediated silencing of ALK5 (Fig. 5H,I, Supporting Fig. S7G,H), thus strengthening the major role of ALK5 in Bmp2 regulation. These results show that ALK5 contributes for the basal expression of Bmp2 and that its activation through TGFβ1 strongly reduces Bmp2 levels in LSECs.

LSEC-secreted BMP2 activates mRNA expression of the iron hormone hepcidin in HCs. To investigate whether increased Bmp2 mRNA levels induced by ALK5 inhibition indeed activate hepcidin, we treated primary HCs alone or in coculture with primary LSECs with or without the ALK5 inhibitor. Only in the coculture setting we observe a significant induction of Bmp2 and hepcidin mRNA levels (Supporting Fig. S8A,B). We next treated wild-type mice with the ALK5 inhibitor LY2157299 (galunisertib) that is currently, at time of publication, in phase I and II of clinical trials for different types of solid cancers (see ClinicalTrials.gov; National Institutes of Health). We show that, analogous to the coculture setting, the acute administration of galunisertib to mice up-regulates Bmp2 and hepcidin mRNA levels in the liver (Supporting Fig. S8C,D). Because both genes can also be regulated by liver iron accumulation, we show that it remains unaltered on ALK5 inhibition (Supporting Fig. S8E). As expected, elevated expression of BMP2 and hepcidin promote iron deposition in the spleen (Supporting Fig. S8F) and a significant reduction of serum iron content (Supporting Fig. S8G) and transferrin saturation (Supporting Fig. S8H). Taken together, we demonstrate that Bmp2 induction on ALK5 inhibition causes hepcidin induction and hypoferremia in cultured cells and in mice.

**Discussion**

Sinusoidal endothelial cells (SECs) are highly specialized endothelial cells hallmarked by an absence of basal lamina and fenestrae that constitute the units of sinusoids. Sinusoids are vessels identified only in liver, spleen, and bone marrow. SECs in the liver, named LSECs, are fundamental for hepatic tissue homeostasis, even though they account only for a small percentage of total liver tissue. Because of the low number of cells and the highly specialized phenotype, protocols for LSEC isolation and culture are extremely challenging. Thus, optimized methods are required to prepare highly pure preparations of primary mouse LSECs. To achieve an efficient yield and purity, most of the available protocols for mouse LSEC isolation require the application of immunoselection, a process that might alter the phenotype of cultured cells.

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Here, we used a method, which is applicable to mouse and rat, wherein the immunoselection step is substituted by selective cell adherence. With this protocol, we isolated mouse and rat LSECs with a very high yield, excellent viability (≈99%), and purity (≈95%).

Cultured LSECs from mice were critical for this study in order to analyze whether cytokines secreted from LSECs exert autocrine effects. We performed ligand/receptor analysis of publicly available RNA-sequencing data from murine LSECs and showed that TGFβ family members are strongly enriched, whereby BMP2, BMP6, and TGFβ1 are the most highly expressed ligands (Fig. 1). Here, we explored the autocrine effects of BMP2, BMP6, and TGFβ1 ligands in LSECs.

Unexpectedly, LSECs do not respond to BMP2 and BMP6 stimulation despite high expression of the respective signaling machinery (receptors and transducers). The unresponsiveness of LSECs to BMPs is partially explained by high levels of the BMP type-I receptor inhibitor FKBP12. However, the less-than-additive effect of BMP6 treatment and FKBP12 inhibition by FK-506 on the expression of BMP target genes suggests that additional mechanisms inhibiting this pathway in LSECs may be operational. Indeed, additional BMP pathway inhibitors are more highly expressed by LSECs compared with HCs, which efficiently respond to BMP2 and BMP6 treatment (Supporting Fig. S3, Fig. 3). We conclude that BMP2 and BMP6, which are induced in conditions where BMP2, BMP6, and TGFβ1 stimulation (Supporting Fig. S3, Fig. 3). We conclude that BMP2 and BMP6, which are induced in conditions of high iron availability in LSECs, predominantly act in a paracrine manner, e.g., in HCs, wherein they induce BMP/SMAD signaling and the expression of downstream target genes, such as the iron hormone hepcidin, which then limits iron release into the bloodstream from the diet and intracellular stores by triggering the degradation of the iron exporter Fpn.

By contrast, TGFβ1 induces a strong response of target genes in LSECs. Importantly, we identified that TGFβ1 reduces Bmp2 mRNA expression through activation of the TGFβ type-I receptor ALK5 in LSECs (Fig. 5). We expect that this result may have multiple implications for ongoing therapeutic studies and contribute to our understanding of various (patho)physiological conditions.

Here, we demonstrate that ALK5 inhibition by galunisertib, a drug currently tested, at time of publication, in clinical trials for solid cancers, increases Bmp2 and hepcidin mRNA expression and causes hypoferremia in mice (Supporting Fig. S8). These findings raise awareness to monitor hypoferremia in patients with cancer who are treated with galunisertib, as patients may experience an aggravation of anemia of cancer because of iron restriction as a treatment side effect.

Our results may have implications for the application of ligand traps for TGFβ cytokines (sotatercept [ACE-011] and luspatercept [ACE-536]). These were originally developed for bone loss disorders and, later on, shown to improve erythropoiesis and ameliorate secondary iron overload in mouse models and patients with β-thalassemia. Inhibition of TGFβ ligands by luspatercept and sotatercept may increase Bmp2 levels and hepcidin expression and, as a consequence, contribute to the reduction of tissue iron accumulation. Along these lines, ALK5 inhibition may be beneficial for patients with the iron overload disorder hereditary hemochromatosis. In support of this idea, we recently showed that acute BMP2 treatment induces hepcidin expression in disease models of hereditary hemochromatosis, a common disorder characterized by primary iron overload due to inappropriately low hepcidin levels. Future experiments will have to assess whether drugs interfering with TGFβ-mediated BMP2 regulation may be applicable for the treatment of iron overload diseases.

Our findings may also be of relevance for understanding molecular mechanisms operational in chronic liver disease (CLD), which is frequently associated with low hepcidin levels and iron overload. The secondary iron overload developed in patients with CLD is associated with more severe disease progression because oxidative stress caused by excess iron accelerates the fibrosis process. We speculate that TGFβ1-mediated Bmp2 reduction (as shown here) may contribute to hepcidin down-regulation in patients with liver fibrosis. Indeed, in contrast to Pai1 and Smad7 (Supporting Fig. S9A,B), hepcidin is predominantly regulated by BMP2 (Supporting Fig. S9C), and its expression is strongly reduced in primary HCs incubated with TGFβ and decreasing concentrations of BMP2 (Supporting Fig. S9D). Further studies in large patient cohorts are needed to validate this hypothesis.

Taken together, we propose that the identification of TGFβ1 as a negative regulator of Bmp2 in LSECs...
offers biological insight into the cross-talk of these pathways in LSECs as well as perspectives for the treatment of hepcidin deficiency diseases, such as hereditary hemochromatosis, β-thalassemia, and CLDs.

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Author Contributions: S.C. designed the project, performed experiments, and wrote the manuscript. S.A. performed the bioinformatics analysis. O.M., A.D., N.K.H., K.M., and S.H. performed experiments. A.D., S.H., and S.D. provided expertise in the TGFβ-related experiments. M.U.M. designed and supervised the project and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.31900/suppinfo.