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Iron-dependent BMP6 Regulation in Liver Sinusoidal Endothelial Cells Is Instructed by Hepatocyte-derived Secretory Signals

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The hepatocytic peptide hormone hepcidin regulates systemic iron homeostasis. Increases in iron levels stimulate hepcidin synthesis to reduce dietary iron uptake and iron release from iron recycling macrophages by blocking iron export into the circulation via ferroportin (Fpn).[1] The liver monitors both plasma and liver iron levels and controls hepcidin transcription by bone morphogenetic protein (BMP)/Son Of Mothers Against Decapentaplegic (SMAD) signaling. Critically, bone morphogenetic protein 6 (BMP6) is released in response to elevated hepatic iron levels,[2] but the underlying mechanisms to control *Bmp6* expression are unresolved.

The growth factor BMP6 was initially identified as a regulator of bone development. However, lack of BMP6 does not affect bone homeostasis, but causes systemic iron overload in mice.[3] Moreover, *BMP6* mutations are associated with iron overload in patients,[4–8] supporting its crucial regulatory function in iron metabolism. In the liver, BMP6 is mainly produced by liver sinusoidal endothelial cells (LSECs). Indeed, specific *Bmp6* deletion in endothelial cells, but not in macrophages or hepatocytes (HCs), causes iron overload in the mouse due to low hepcidin levels.[9] Previous data suggest that the iron-dependent *Bmp6* induction in LSECs is cell autonomous[10–12] and mediated by the activation of the antioxidant nuclear factor Nuclear factor erythroid 2-related factor 2 (NRF2) via intracellular iron accumulation.[12] This model is not supported by the analysis of LSECs isolated from mice with hemochromatosis (*Hjv* knockout [KO]), hallmarked by elevated *Bmp6* levels but iron-deficient molecular signature in LSEC.[13] Here, we systematically dissect the mechanism of BMP6 control in response to liver iron overload and reveal that a factor produced by HCs is required for *Bmp6* induction in LSECs.

We first explored the requirement of iron accumulation in LSECs for increased *Bmp6* messenger RNA (mRNA) expression in vivo by comparing wild-type (wt) mice maintained on a "high iron" diet' (Fe^{high} diet)[14] and Fpn(C326S) mice exerting iron overload due to a point mutation in Fpn that disrupts hepcidin binding[15] (Suppl. Figure S1A–H). From these mice, we isolated HCs and LSECs and analyzed ferritin L protein and *transferrin receptor* (*Tfr*) 1 mRNA levels, as markers of intracellular iron content. HCs and LSECs isolated from Fe^{high} diet mice show a molecular signature consistent with intracellular iron accumulation, with elevated ferritin protein and reduced *Tfr1* mRNA levels (Suppl. Figure S1I–L; Figure 1B). Similarly, Fpn(C326S) mice show iron-loaded HCs. However, LSECs express similar levels of ferritin and increased *Tfr1* mRNA in comparison with wt controls (Suppl. Figure S1I, J; Suppl. Figure S1M, N; Figure 1C); indicating that they are not iron-loaded but rather iron-deficient. We hypothesize that, in LSECs isolated from Fpn(C326S) mice, iron import via TfR1 (Figure 1A) and the non-transferrin bound iron importers is not sufficient to compensate for the increased Fpn-mediated iron export. Despite the absence of iron overload in LSECs isolated from Fpn(C326S) mice, *Bmp6* mRNA expression is elevated in total liver (Suppl. Figure S1B, F) and purified HCs and LSECs (Figure 1E, F) from both mouse models. These findings extend and support data reported in *Hjv* KO mice by Rausa et al.[13] This strongly suggests that iron accumulation in LSECs is not the driving force for *Bmp6* induction.

Among the different hepatic cell types, LSECs express the highest levels of *Bmp6* (Figure 1D) and the biological relevance of this was demonstrated by endothelial cell-specific *Bmp6* deletion.[9] Available LSEC cell lines are unable to recapitulate all features of primary LSECs.[16] We therefore aimed to validate data obtained in mice in a primary culture of mouse LSECs. As previously shown, this cell preparation is highly pure and maintains fenestrae, denoting lack of cell trans-differentiation.[14] Exposure of LSECs to iron nitrilotriacetate (FeNTA) decreases *Tfr1* mRNA expression and activates *hemoxygenase1* (*Ho1*) transcription (Figure 1G, H), indicating that intracellular iron accumulation induces oxidative stress (Figure 1G). By contrast, *Bmp6* transcription was not induced (Figure 1I). Similar results were obtained in primary mouse LSECs incubated with ferric ammonium citrate (FAC) (Suppl. Figure S2). This finding supports our data obtained

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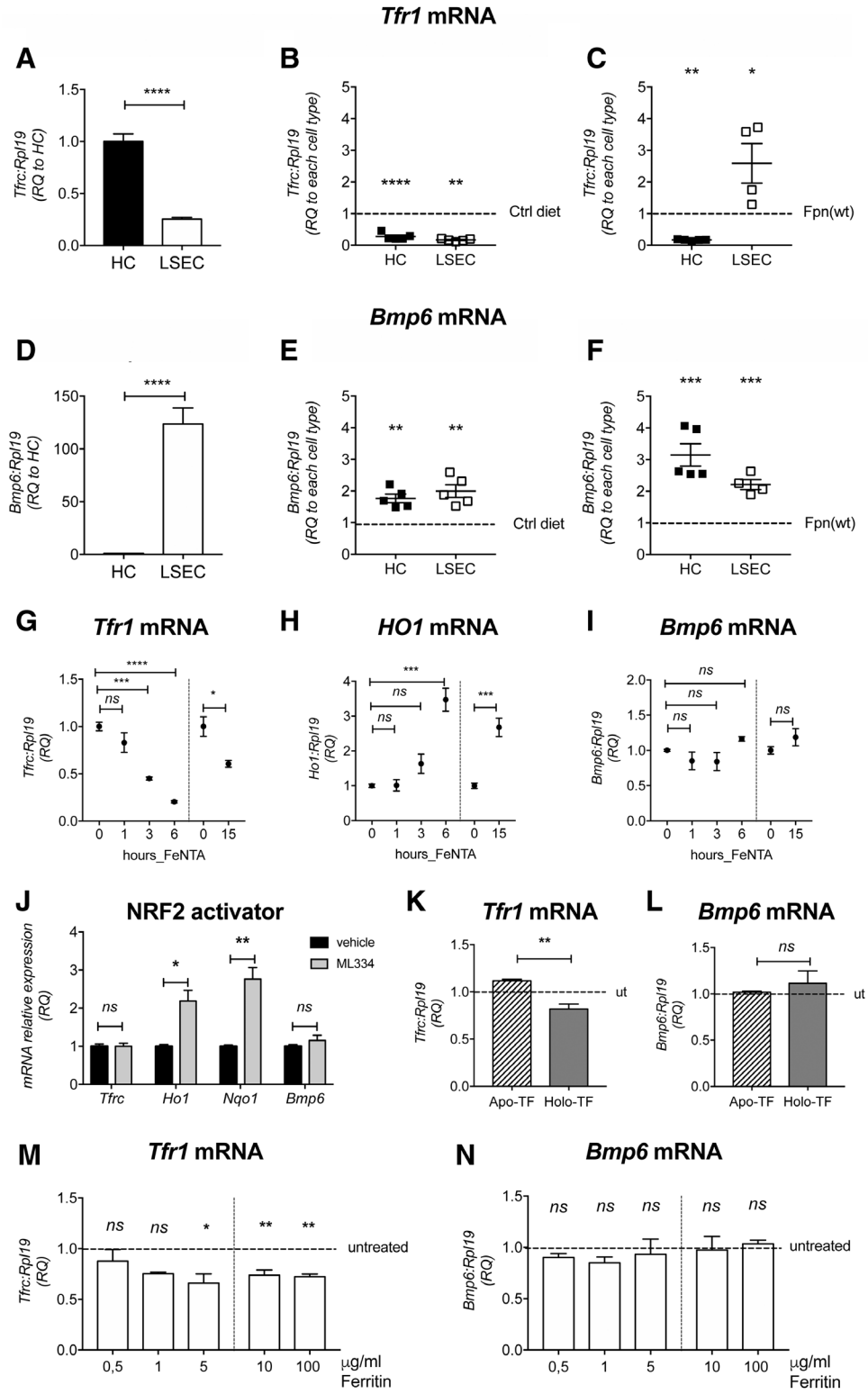

Figure 1. Iron accumulation in LSECs is not a prerequisite for an iron-dependent increase of Bmp6 mRNA expression. (A–F), Total RNA was extracted from HCs and LSECs isolated from male wt mice maintained either on an iron-balanced or iron-loaded (Fe^{high}) diet for 4 wk or from male Fpn(C326S) mice and Fpn(wt) controls. All groups were sacrificed at 11 wk of age. Primary mouse LSECs were serum starved 2 h and then incubated with (G–I) 50 μM of FeNTA for 1, 3, 6, and 15 h and with (J) 20 μM of ML334 for 8 h or left untreated. Freshly prepared LSECs were serum starved for 2 h and then left untreated or incubated with (K and L) 30 μM of Apo-TF or Holo-TF for 18 h and (M and N) increasing concentration of ferritin (0.5 to 100 μg/mL). mRNA expression of (A–C, G, J, K, M) *Tfr1* (*Tfrc*), (D–F, I, J, L, N) *Bmp6*, (H, J) *Ho1*, and (J) *Nqo1* was analyzed by qRT-PCR and normalized to the housekeeping gene *Rpl19*. All treatments were performed in serum-free medium. A minimum of 3 biological replicates per condition is shown. A minimum of 2 independent experiments with at least 3 biological replicates was performed. In (G–I, M and N) results of 2 independent experiments are shown and they are divided by a dashed line. Data are reported as mean ± SEM and represented as (A, D) relative expression to HC (B and C, E and F) to control mice or (G–N) to vehicle treated or untreated cells (as indicated with the dashed line). Two-tailed Student *t* test and 1-way ANOVA were calculated: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Apo-TF = Apo-transferrin; BMP6 = bone morphogenetic protein 6; Fe^{high} = high iron; FeNTA = iron nitrilotriacetate; Fpn = ferroportin; HC = hepatocyte; *Ho1 = hemoxygenase1*; Holo-TF = Holo-transferrin; LSECs = liver sinusoidal endothelial cells; mRNA = messenger RNA; ns = not significant; qRT-PCR = quantitative real time PCR; RQ = relative quantification; *Tfr = transferrin receptor*; wt = wild-type.

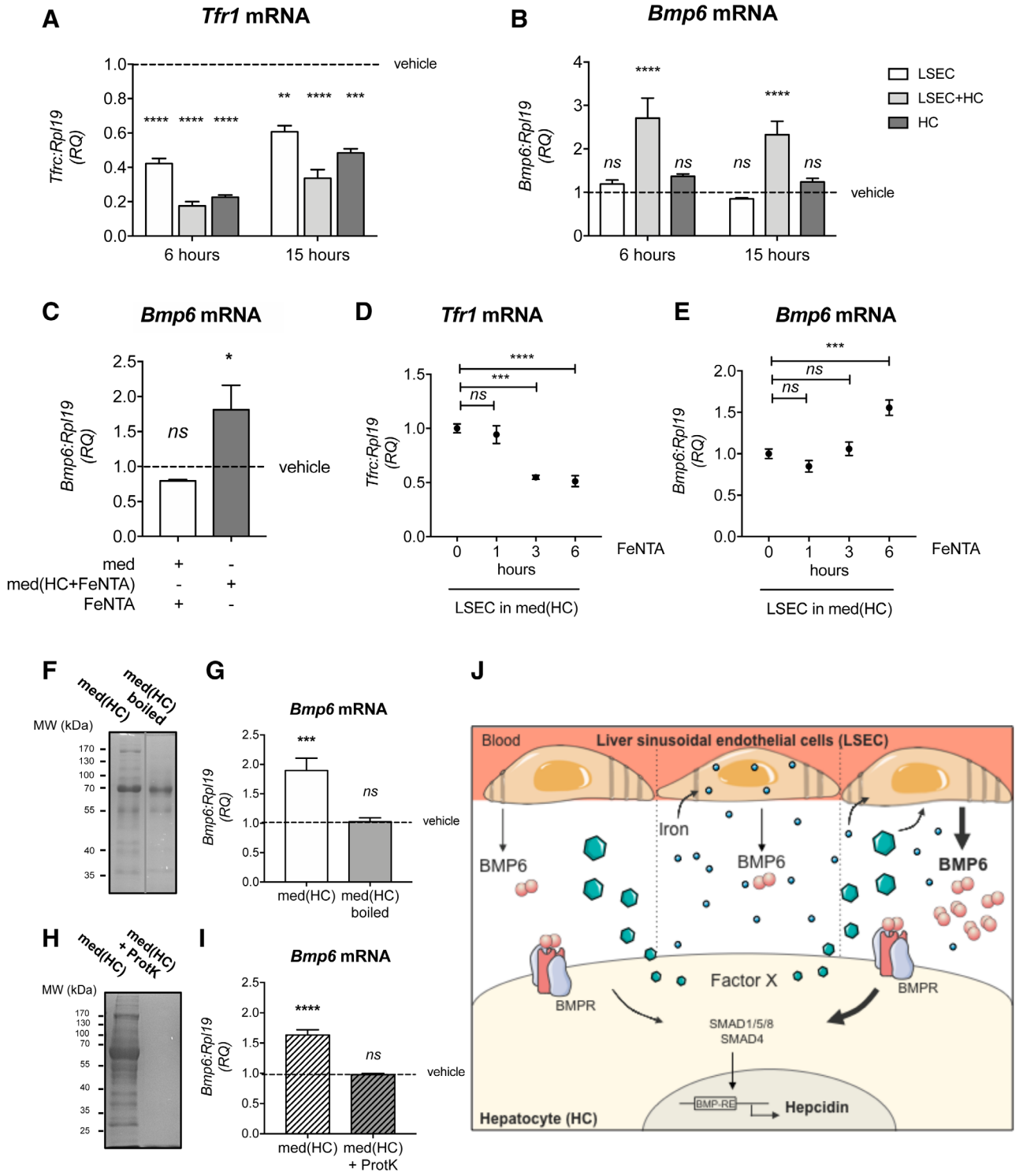

Figure 2. Crosstalk between primary LSECs and HCs is critical to induce Bmp6 mRNA levels in response to iron. (A and B), Primary mouse LSECs and HCs were maintained together (LSEC + HC) or separately and incubated with 50 µM of FeNTA for 6 and 15 h. The 2 time points represent 2 independent experiments. (C), Freshly prepared primary mouse LSECs were treated with unconditioned medium (med) supplemented or not with 50 µM of FeNTA for 6 h, or with medium from HC primary culture treated or not with 50 µM of FeNTA for 18 h (med(HC + FeNTA)). (D and E), Primary mouse LSECs were cultivated in HC derived medium, whereby HC remained untreated (med(HC)). Fifty µM of FeNTA was then added for 1, 3, and 6. (F and G), Hepatocyte conditioned medium (med(HC)) was heat-inactivated at 95°C, 30 min, and insoluble proteins were precipitated by high-speed centrifugation. (F), Coomassie blue staining of med(HC) boiled or unboiled. (G), Primary mouse LSECs were incubated with untreated or heat-inactivated med(HC) supplemented or not with 50 µM FeNTA. (H and I), Proteinase K was immobilized on agarose resin and used to digest proteins secreted in the med(HC). (H), Proteinase K treated and untreated medium was loaded on a SDS-PAGE and stained with Coomassie blue or (I) used to cultivate primary LSECs in presence or absence of 50 µM FeNTA. Total RNA was extracted and mRNA expression of (A, D) *Tfr1* (*Tfrc*) and (B, C, E, G, I) *Bmp6* was analyzed by qRT-PCR. Gene expression was normalized to the housekeeping gene *Rpl19*. All treatments were performed in serum-free medium. A minimum of 3 biological replicates per condition is shown. A minimum of 2 independent experiments with at least 3 biological replicates was performed. (J), Schematic representation of BMP6 regulation in response to iron. LSEC-secreted BMP6 induces hepcidin by activating SMAD1/5/8 signaling pathway in hepatocytes. The combination of a ubiquitously secreted hepatocyte molecule and iron induces BMP6 in LSECs that, subsequently, contributes to hepcidin upregulation. Data are represented as relative quantification to vehicle treated cells and reported as mean ± SEM. Two-tailed Student *t* test and 2-way ANOVA were calculated: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. BMP6 = bone morphogenetic protein 6; BMPR = bone morphogenetic protein receptor; BMP-RE = bone morphogenetic protein responsive element; FeNTA = iron nitrilotriacetate; HC = hepatocyte; LSECs = liver sinusoidal endothelial cells; med = medium; mRNA = messenger RNA; MW = molecular weight; ns = not significant; ProtK = proteinase K; qRT-PCR = quantitative real time PCR; RQ = relative quantification; SDS-PAGE = sodium dodecyl sulphate - polyacrylamide gel electrophoresis; SMAD = Son Of Mothers Against Decapentaplegic; *Tfr = transferrin receptor.*

in Fpn(C326S) mice and suggests that iron accumulation in LSECs may not be a direct signal activating *Bmp6* in an oxidative stress-dependent manner, as previously proposed.[12] To analyze whether the antioxidant transcription factor NRF2 controls *Bmp6* expression, we applied ML334, a drug promoting NRF2 activation. We observed induction of NRF2 target genes (*Ho1*; *Nqo1*); however, mRNA expression of *Tfr1* and *Bmp6* remained unchanged (Figure 1J). These data demonstrate that NRF2 activation by iron and ML334 does not activate *Bmp6* transcription in freshly prepared primary mouse LSECs.

We next explored whether different iron sources can elicit a BMP6 response. Transferrin saturation increases to approximately 100% in Fpn(C326S) mice.[15] However, holo-transferrin (holo-Tf) treatment of LSECs did not activate *Bmp6* expression (Figure 1K, L), suggesting that a very high transferrin saturation does not contribute to *Bmp6* upregulation. In addition, delivery of iron in the form of ferritin did not increase *Bmp6* mRNA levels, despite eliciting *Tfr1* reduction in a dose-dependent manner (Figure 1M, N). This indicates that the previously reported treatment of mice with ferritin[17] may have induced *Bmp6* expression levels in LSECs indirectly. Taken together, these findings suggest that the iron-dependent *Bmp6* regulation in LSECs is not cell autonomous.

In contrast to LSECs, HCs accumulate iron in Fe^{high} and Fpn(C326S) mice (Suppl. Figure S1I–N; Figure 1B, C). Given the spatial proximity of LSECs and HCs and reports of their reciprocal cross-talk, we hypothesized that HCs may be required for correct iron sensing and subsequent *Bmp6* induction in LSECs. Therefore, we cultivated primary mouse LSECs and HCs, either alone or in co-culture, and treated with iron (Figure 2A, B). Remarkably, *Bmp6* is only induced in the co-culture setting (Figure 2B). We next explored if the secretome of iron-loaded HCs is sufficient to induce *Bmp6*. Therefore, we incubated LSECs with the supernatant of a monoculture of HCs that was treated or not with iron (med(HC)). As controls, LSECs were also cultivated with unconditioned medium supplemented or not with iron (med). Importantly, we observed *Bmp6* induction when LSECs were incubated with conditioned medium derived from iron-loaded HCs (Figure 2C), clearly demonstrating that molecule(s) released by iron-loaded HCs are sufficient to induce *Bmp6* mRNA expression in LSECs. Of note, mice lacking the non-transferrin-bound iron importer Zrt- and Irt-like Protein 14 (ZIP14) and that are maintained on a Fe^{high} diet do not accumulate iron in HCs. Despite this, hepatic *Bmp6* levels are increased,[18] suggesting that increased iron content in HCs may not be essential to observe *Bmp6* upregulation in vivo. Therefore, we investigated whether the HC molecule(s) required for the iron-dependent *Bmp6* induction in LSECs are secreted by HCs under steady state conditions, that is, in the absence of iron. LSECs were incubated with conditioned medium derived from untreated HCs, whereby iron (FeNTA) was added retrospectively to the supernatant. Iron import is not affected by adding HC secretome to LSECs, as *Tfr1* mRNA expression is reduced (Figure 2D). Notably, *Bmp6* is induced 6 hours after iron treatment (Figure 2E). Similar data were observed by replacing primary mouse HCs with the murine HC cell line Hepa1-6 (Suppl. Figure S3A–F). In contrast, the combined treatment of HC secretome and holo-Tf did not alter *Bmp6* mRNA expression in primary LSECs (Suppl. Figure S3G); this suggests that only an unbound form of iron synergizes with HC-released molecules for the regulation of *Bmp6*. To explore if the factor released by HCs is a protein, we subjected the conditioned medium obtained from an HC monoculture to heat-inactivation, a process causing heat-dependent protein denaturation and precipitation, or to proteinase K digestion. Both treatments strongly reduced the amount of total protein within the HC-conditioned medium (Figure 2F, H). Importantly, when LSECs were exposed to the protein-depleted medium, *Bmp6* induction in the presence of iron was prevented (Figure 2G, I).

In conclusion, our findings demonstrate that in contrast to previous reports,[10–12] the iron-dependent *Bmp6* regulation in LSECs is not cell autonomous. None of the tested iron sources, such as FeNTA, FAC, holo-Tf, or ferritin induced *Bmp6* mRNA expression in primary culture of LSECs. We expect freshly prepared primary mouse LSECs used in this study to be superior in reflecting the iron response observed in vivo compared with endothelial cell lines or commercially available primary LSECs. By establishing a co-culture system of primary mouse LSECs and HCs, we show that cell-to-cell communication between HCs and LSECs is essential for iron sensing and subsequent *Bmp6* regulation. Specifically, we demonstrate that proteins secreted by HCs induce *Bmp6* in LSECs when combined with iron treatment (Figure 2J). This study uncovers a novel regulatory step of iron metabolism and establishes a basis for the future identification of regulators of the BMP6-hepcidin axis. Our results unravel a novel model of iron-dependent BMP6 regulation where HCs secrete protein(s) that function as BMP6 activator in the presence of iron. This model explains data from genetically modified mice, demonstrating that intracellular iron accumulation in both, HCs and LSECs, is not essential for increased BMP6 expression in response to iron overload. Finally, we hypothesize that component(s) of the HC secretome interact with iron, possibly in the noncellular space between HCs and LSECs (space of Disse), functioning as BMP6 activator(s).

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AUTHOR CONTRIBUTIONS

SC and MUM involved in conceptualization. SC, SA, OM, KM, and ARA involved in investigation. SC involved in visualization. SC and MUM involved in writing—original draft. SA, OM, and MWH involved in writing—review & editing. MWH and MUM involved in supervision. MUM involved in funding acquisition.

DISCLOSURES

MUM is a *HemaSphere* Associate Editor. All the other authors have no conflicts of interest to disclose.

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REFERENCES

1. Muckenthaler MU, Rivella S, Hentze MW, et al. A red carpet for iron metabolism. *Cell*. 2017;168:344–361.
2. Colucci S, Marques O, Altamura S. 20 years of Hepcidin: how far we have come. *Semin Hematol*. 2021;58:132–144.
3. Meynard D, Kautz L, Darnaud V, et al. Lack of the bone morphogenetic protein BMP6 induces massive iron overload. *Nat Genet*. 2009;41:478–481.
4. Daher R, Kannengiesser C, Houamel D, et al. Heterozygous mutations in BMP6 pro-peptide lead to inappropriate hepcidin synthesis and moderate iron overload in humans. *Gastroenterology*. 2016;150:672–683.e4.
5. Piubelli C, Castagna A, Marchi G, et al. Identification of new BMP6 pro-peptide mutations in patients with iron overload. *Am J Hematol*. 2017;92:562–568.
6. McDonald CJ, Rishi G, Secondes ES, et al. Evaluation of a bone morphogenetic protein 6 variant as a cause of iron loading. *Hum Genomics*. 2018;12:23.
7. Alvarenga AM, da Silva NK, Fonseca PFS, et al. Novel mutations in the bone morphogenetic protein 6 gene in patients with iron overload and

non-homozygous genotype for the HFE p.Cys282Tyr mutation. *Blood Cells Mol Dis*. 2020;84:102444.
8. Tchernitchko D, Lamoril J. Novel BMP6 gene mutation in patient with iron overload. *Eur J Gastroenterol Hepatol*. 2021;33:1029–1030.
9. Canali S, Zumbrennen-Bullough KB, Core AB, et al. Endothelial cells produce bone morphogenetic protein 6 required for iron homeostasis in mice. *Blood*. 2017;129:405–414.
10. Wang S, Chen C, Yu L, et al. Bone morphogenetic protein 6-mediated crosstalk between endothelial cells and hepatocytes recapitulates the iron-sensing pathway in vitro. *J Biol Chem*. 2021;297:101378.
11. Noguchi T, Ikeda M, Murakami M, et al. Regulatory expression of bone morphogenetic protein 6 by 2,2'-dipyridyl. *Biochim Biophys Acta Gen Subj*. 2020;1864:129610.
12. Lim PJ, Duarte TL, Arezes J, et al. Nrf2 controls iron homeostasis in haemochromatosis and thalassaemia via Bmp6 and hepcidin. *Nat Metab*. 2019;1:519–531.
13. Rausa M, Pagani A, Nai A, et al. Bmp6 expression in murine liver non parenchymal cells: a mechanism to control their high iron exporter activity and protect hepatocytes from iron overload? *PLoS One*. 2015;10:e0122696.
14. Colucci S, Altamura S, Marques O, et al. Liver sinusoidal endothelial cells suppress bone morphogenetic protein 2 production in response to TGFβ pathway activation. *Hepatology*. 2021;74:2186–2200.
15. Altamura S, Kessler R, Gröne HJ, et al. Resistance of ferroportin to hepcidin binding causes exocrine pancreatic failure and fatal iron overload. *Cell Metab*. 2014;20:359–367.
16. Poisson J, Lemoinne S, Boulanger C, et al. Liver sinusoidal endothelial cells: physiology and role in liver diseases. *J Hepatol*. 2017;66:212–227.
17. Feng Q, Migas MC, Waheed A, et al. Ferritin upregulates hepatic expression of bone morphogenetic protein 6 and hepcidin in mice. *Am J Physiol Gastrointest Liver Physiol*. 2012;302:G1397–G1404.
18. Jenkitkasemwong S, Wang CY, Coffey R, et al. SLC39A14 is required for the development of hepatocellular iron overload in murine models of hereditary hemochromatosis. *Cell Metab*. 2015;22:138–150.