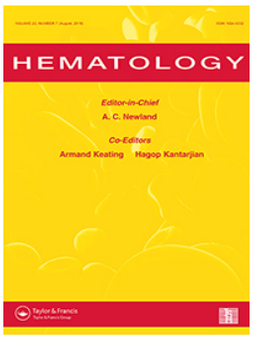

Taylor & Francis
Taylor & Francis Group

Hematology

ISSN: (Print) 1607-8454 (Online) Journal homepage: https://www.tandfonline.com/loi/yhem20

Modulation of hepcidin expression by normal control and beta0-thalassemia/Hb E erythroblasts

Janejira Jaratsittisin, Wannapa Sornjai, Saovaros Svasti, Suthat Fucharoen, Sittiruk Roytrakul & Duncan R. Smith

To cite this article: Janejira Jaratsittisin, Wannapa Sornjai, Saovaros Svasti, Suthat Fucharoen, Sittiruk Roytrakul & Duncan R. Smith (2018) Modulation of hepcidin expression by normal control and beta0-thalassemia/Hb E erythroblasts, Hematology, 23:7, 423-428, DOI: 10.1080/10245332.2017.1405571

To link to this article: https://doi.org/10.1080/10245332.2017.1405571

HEMATOLOGY
2018, VOL. 23, NO. 7, 423–428
https://doi.org/10.1080/10245332.2017.1405571

Taylor & Francis
Taylor & Francis Group

THALASSEMIA

Modulation of hepcidin expression by normal control and beta0-thalassemia/Hb E erythroblasts

Janejira Jaratsittisin [a], Wannapa Sornjai [a], Saovaros Svasti [a], Suthat Fucharoen [a], Sittiruk Roytrakul [b] and Duncan R. Smith [a]

[a]Institute of Molecular Biosciences, Mahidol University, Salaya, Thailand; [b]National Science and Technology Development Agency, National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathum Thani, Thailand

ABSTRACT

Objectives: The inherited genetic disorder beta0-thalassemia/Hb E disease is associated with the over-suppression of the master regulator of iron homeostasis, the peptide hormone hepcidin. How developing erythroid cells mediate the suppression of hepcidin remains controversial, although a number of inhibitors have been proposed.

Methods: To investigate the ability of erythroid cells to suppress hepcidin expression in liver cells, conditioned media from the culture of *in vitro* differentiating erythroblasts (from normal controls and beta0-thalassemia/Hb E patients) was used to treat HepG2 cells, and the effects on hepcidin expression were assayed by real-time quantitative PCR and confocal microscopy.

Results: Early activation followed by later suppression of hepcidin expression was seen posttreatment. Markedly, however, no significant differences were observed between suppression of hepcidin as mediated by media from the culture of erythroblasts from normal controls and beta0-thalassemia/Hb E patients

Discussion: Previous studies investigating the suppression of hepcidin expression in beta0-thalassemia/Hb E disease have used patient-derived serum samples, which are complex fluids with contributions from multiple cell types. This study has developed a simple *in vitro* system that allows investigation of how a single cell type mediates hepcidin expression. The results support proposals that over-suppression of hepcidin seen in beta-thalassemia/Hb E patients is a consequence of the increased mass of erythropoiesis and not defects in the signaling process per se.

Conclusion: The *in vitro* cell system developed here allows further investigation into the processes mediating erythroid cell suppression of liver hepcidin expression in both normal and pathological states.

KEYWORDS

Hepcidin; beta-thalassemia; liver; erythroblasts; suppression; erythroferrone; growth differentiation factor 15; twisted gastrulation protein

Introduction

Iron is an essential element for a number of critical biological processes including cellular respiration and oxygen transport [1]. In the absence of an iron excretion system in humans, iron homeostasis is carefully maintained as both iron overload and iron deficiency can have severe consequences [2]. The 25-amino acid, 2789.8 Da peptide hormone hepcidin (also known as liver-expressed antimicrobial peptide 1) is the master regulatory control peptide for iron homeostasis [3]. Hepcidin is predominantly produced in the liver where it is initially translated as an 84-amino-acid-long pre-pro-peptide which is subsequently processed to generate the 25-amino acid mature peptide hormone [4], which possesses a beta-hairpin structure containing four disulfide bonds [5]. Six N-terminal amino acids are highly conserved and essential for hepcidin activity [6]. Hepcidin is predominantly produced by hepatocytes, and the mature peptide hormone is released into the circulation either as a free form or weakly bound with albumin and alpha$_2$-macroglobulin [7].

Hepcidin acts through its interaction with its receptor protein, ferroportin, which is the only known iron exporter protein located on the plasma membrane of professional iron exporting cells such as duodenal enterocytes, hepatocytes, macrophages, spleen cells [8] and erythroid precursor cells [9]. When hepcidin binds to ferroportin, it promotes the internalization and subsequent degradation of the ferroportin/hepcidin complex, inhibiting the ability of these cells to export iron [10]. When hepcidin expression is high, export of iron from storage cells is low due to the internalization of ferroportin in iron storage cells, and conversely, when hepcidin expression is low, iron storage cells can release iron into the system.

The hepcidin–ferroportin interaction plays a role in systemic iron regulation in three main ways, by regulating dietary iron absorption of duodenal enterocytes, by regulating iron storage in hepatocytes and by

CONTACT Duncan R. Smith duncan_r_smith@hotmail.com Institute of Molecular Biosciences, Mahidol University, Salaya Campus, 25/25 Phuttamonton Sai 4, Salaya, Nakhon Pathom 73170, Thailand

Supplemental data for this article can be accessed at https://doi.org/10.1080/10245332.2017.1405571

regulating iron recycling from senescent erythrocytes in the spleen [11]. Apart from regulation by the plasma iron concentration, hepcidin production is also directly regulated by erythropoietic activity in the bone marrow [12]. Increased erythropoiesis suppresses hepcidin production, resulting in a greater supply of iron from duodenal absorption and iron storage release, to allow sufficient iron to be available for hemoglobin synthesis. However, the molecular mechanisms mediating hepcidin suppression are not well understood. It was proposed that during erythropoiesis, proteins secreted by erythroid progenitor cells suppress hepcidin production from the liver [13], and a number of mediators of this suppression have been proposed, including growth differentiation factor 15 (GDF15) [14], twisted gastrulation protein (TWSG1) [15] and erythroferrone [16].

Beta0-thalassemia/Hb E is a compound-inherited disorder, deriving from the co-inheritance of a mutation in one allele of the beta-globin gene and the structural hemoglobin HbE variant in the second allele [17]. Even after allowing for additional modifying factors such as co-inheritance of alpha-hemoglobinopathies, presentation of this disease is remarkably varied, ranging from mild anemia to a severe, transfusion-dependent anemia [18]. The unbalanced production of alpha-globin chains leads to the death of the developing erythroblast at the polychromatic normoblast stage in a process termed ineffective erythropoiesis [19]. The resultant anemia leads to expansion of the erythroid mass, and it has been proposed, based on studies in mice, that the subsequently increased secretion of erythroferrone results in the decreased hepcidin expression observed in beta-thalassemia patients [16,20].

However, previous studies investigating erythroid factors that mediate hepcidin suppression were based on analysis of plasma of beta-thalassemia patients [14] and on studies undertaken in mice [15,16], and plasma is known as a complex fluid whose composition is influenced by many cells [21]. To address this, we sought to develop an experimental system that would allow investigation of suppression of hepcidin expression by developing erythroblasts. Cells from both normal controls and from beta0-thalassemia/Hb E patients were investigated.

Materials and methods

Erythroid cell culture

The study was performed in accordance with the Helsinki Declaration and was conducted after approval by the Ethical Committee, Mahidol University Institutional Review Board. Written informed consent was obtained from all subjects. Patients were identified as described previously [22] and were classified for severity according to the severity scale described previously [23]. Normal controls were screened as described previously [22].

CD34+ hematopoietic stem cells were collected and cultured under conditions driving erythropoiesis as described previously [22]. Briefly, peripheral blood mononuclear cells were isolated from 25 mL (patients) or 50 mL (controls) of venous blood by layering over Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway) and subsequently CD34+ cells were selected by magnetic bead isolation (Miltenyi Biotech, Auburn, CA, USA). Cells were cultured in Iscove's modified Dulbecco medium (IMDM; Gibco BRL, Gaithersburg, MD, USA) containing 15% heat-inactivated fetal bovine serum (FBS; Gibo BRL), 15% human AB serum, 2 U/mL recombinant human (rHu) erythropoietin (Janssen-Cilag Ltd, Auckland, New Zealand), 20 ng/mL rHu stem cell factor (Cell Signaling Technology, Danvers, MA, USA), and 10 ng/mL rHu interleukin-3 (IL-3; Promokine, Heidelberg, Germany) at 37°C with 5% CO_2. Media was replaced with fresh culture media without rHu IL-3 on Days 3, 7 and 10. As reported previously, this methodology produces 85% CD34+ cells and more than 90% proerythroblasts on Day 7 of culture [22]. Normal cell numbers are roughly 1×10^5, 5×10^5, 1×10^6 and 5×10^6on Days 3, 7, 10 and 14, respectively, but as reported previously, cells from thalassemia patients expand to a greater degree [22] and additionally show an accelerated differentiation [24].

Conditioned media treatment

HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. These cells were trypsinized and seeded into 6-well culture plates at a density of 2×10^6 cells per well, followed by incubation at 37°C with 5% CO_2 for 24 hours. A549 cells (ATCC CCL-185; human lung epithelial cell line) were cultured in MEM (HyClone, Thermo Fisher Scientific Inc., Waltham, MA, USA) with 5% FBS at 37° C with 5% CO_2.

Media used for the culture of normal control, mild and severe beta0-thalassemia/Hb E erythroblasts (conditioned media) were pooled (minimum $n = 5$). Control media was complete IMDM incubated for the same time and under the same conditions as the experimental media. HepG2 cells were treated with 2 mL of the pooled conditioned media or control media and incubated at 37°C with 5% CO_2 for 0, 3, 6, 12, 24, 36 and 48 hours, after which the treated cells were collected for determination of hepcidin expression by quantitative PCR. Experiments were performed independently in triplicate.

Cell viability assay

The cytotoxicity of erythroblast conditioned media towards HepG2 cells was assessed by trypan blue

staining. HepG2 cells were treated for 24 and 48 hours with Day 3, 7, 10 and 14 conditioned media derived from culture of erythroblasts from normal controls, mild and severe beta0-thalassemia/ Hb E patients. The treated cells were subsequently trypsinized with 0.25% trypsin-EDTA and stained with trypan blue, and cell viability was established using a hemocytometer under a microscope. HepG2 cells cultured in parallel under standard conditions were evaluated as controls.

Quantitative RT-PCR

Total RNA was extracted from the treated HepG2 cells and cultured erythroid cells using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). The extracted RNA was treated with DNase I (Promega, Madison, WI, USA) at 37°C for 1 hour and used as a template for cDNA synthesis using RevertAid Reverse Transcriptase (Thermo Fisher Scientific Inc.) with an oligo dT primer (Bio Basic, Inc., Amherst, NY, USA). To determine mRNA expression levels, quantitative RT-PCR (qRT-PCR) was performed using the KAPA® SYBR FAST qPCR Kit 2X Master Mix (Kapa Biosystems Inc., Wilmington, MA, USA) with specific primers and beta-actin was used as a reference gene. Primers used were: HAMP-F: 5′-ATGGCACTGAGCTCCCAGAT-3′, HAMP-R: 5′-TTCTACGTCTTGCAGCACATCCC-3′, beta-actin-F: 5′-GAAGATGACCCAGATCATGT-3′, beta-actin-R: 5′-ATCTCTTGCTCGAAGTCCAG-3′, GDF-15-F: 5′-CTCCAGATTCCGAGAGTTGC-3′, GDF-15-R: 5′-AGAGATACGCAGGTGCAGGT-3′, TWSG1-F: 5′-GCCCAAAGTGCTGACATAGG-3′, TWSG1-R: 5′-CAAGCTGTTTCCTGGTGCTC-3′, ERFE-F: 5′-ACGAGCTTGGCGTCTACTAC-3′, ERFE-R: 5′-GACTGGATGCAGATGAGCAG-3′. The relative hepcidin expression was normalized against beta-actin, followed by normalization against the control media (complete IMDM) treated HepG2 cells and was calculated using the following equations: $\Delta\Delta Ct = \Delta Ct$ (case) − ΔCt (control), ΔCt (case) = Ctcase (HAMP) − Ct case (Actin), ΔCt (control) = Ct control (HAMP) − Ct control (Actin). Experiments were performed independently in triplicate.

Indirect immunofluorescence assay

HepG2 cells were grown on coverslips in 6-well plates seeded with 8×10^5 cells/well and incubated at 37°C with 5% CO_2 for 24 hours, following which cells were treated with 2 mL conditioned media from the culture of Day 14 normal control erythroblasts, 2 mL of conditioned media from the culture of A549 cells or 2 mL of DMEM or complete IMDM. At 6 and 24 hours posttreatment, cells were fixed with 4% paraformaldehyde for 20 minutes and then permeabilized with 0.3% Triton X-100 in 1X PBS for 10 minutes at room temperature and washed twice with 0.03% Triton X-100 in 1× PBS. Cells were then blocked with 5% BSA in 1× PBS for 30 minutes and washed again. After that, cells were incubated with a 1:10 dilution of a rabbit polyclonal anti-hepcidin antibody (Thermo Fisher Scientific Inc.) in 0.03% Triton X-100 in 1× PBS overnight. Cells were washed subsequently three times and incubated with a 1:50 dilution of donkey anti-rabbit Alexa Flour 647 antibody in 0.03% Triton X-100 in 1× PBS and a 1:500 dilution of DAPI in 0.03% Triton X-100 in 1× PBS for 2 hours. After incubation, the cells were washed three times as previously before mounting with Prolong. Finally, slides were observed under a Carl Zeiss Laser Scanning Confocal Microscope (LSM 800).

Statistical analysis

Statistical analysis was performed using the PASW statistics 18 (SPSS Inc. Chicago, IL, USA). The relative hepcidin expression from three independent replicates analyzed in triplicate was compared using independent sample *t*-tests. A *p* value of less than .05 was considered as a statistically significant.

Results

Evaluation of suitability of HepG2 cells for hepcidin expression suppression studies

We initially evaluated the suitability of the HepG2 cell line to serve as the basis for a system for investigating the mechanism of suppression of hepcidin expression by developing erythroblasts. Expression of hepcidin by HepG2 cells was therefore evaluated by RT-PCR. Results (data not shown) showed that HepG2 cells expressed detectable levels of the hepcidin message. We next determined whether the media in which erythroblasts had been cultured was cytotoxic to HepG2 cells. In our standard culture methodology for erythroid cell differentiation, CD34+ cells are selected through a magnetic bead isolation method and then these cells are differentiated into erythroid precursor cells through addition of appropriate cytokines, and the media is changed on Days 3, 7, 10 and 14 [22]. Thus, the media removed on those days was defined as conditioned media, and designated as the day on which it was removed (e.g. Day 3 conditioned media had been used to culture initial CD34+ cells from Day 1 to Day 3). HepG2 cells were therefore incubated with Day 3, 7, 10 and 14 conditioned media that was derived from culturing erythroblasts of normal controls as well as erythroblasts from mild and severe beta0-thalassemia/Hb E patients for 24 hours, after which cell viability was assessed by the trypan blue exclusion test. Results (Supplemental Figure 1) showed no significant cytotoxicity of erythroblast conditioned media towards HepG2 cells.

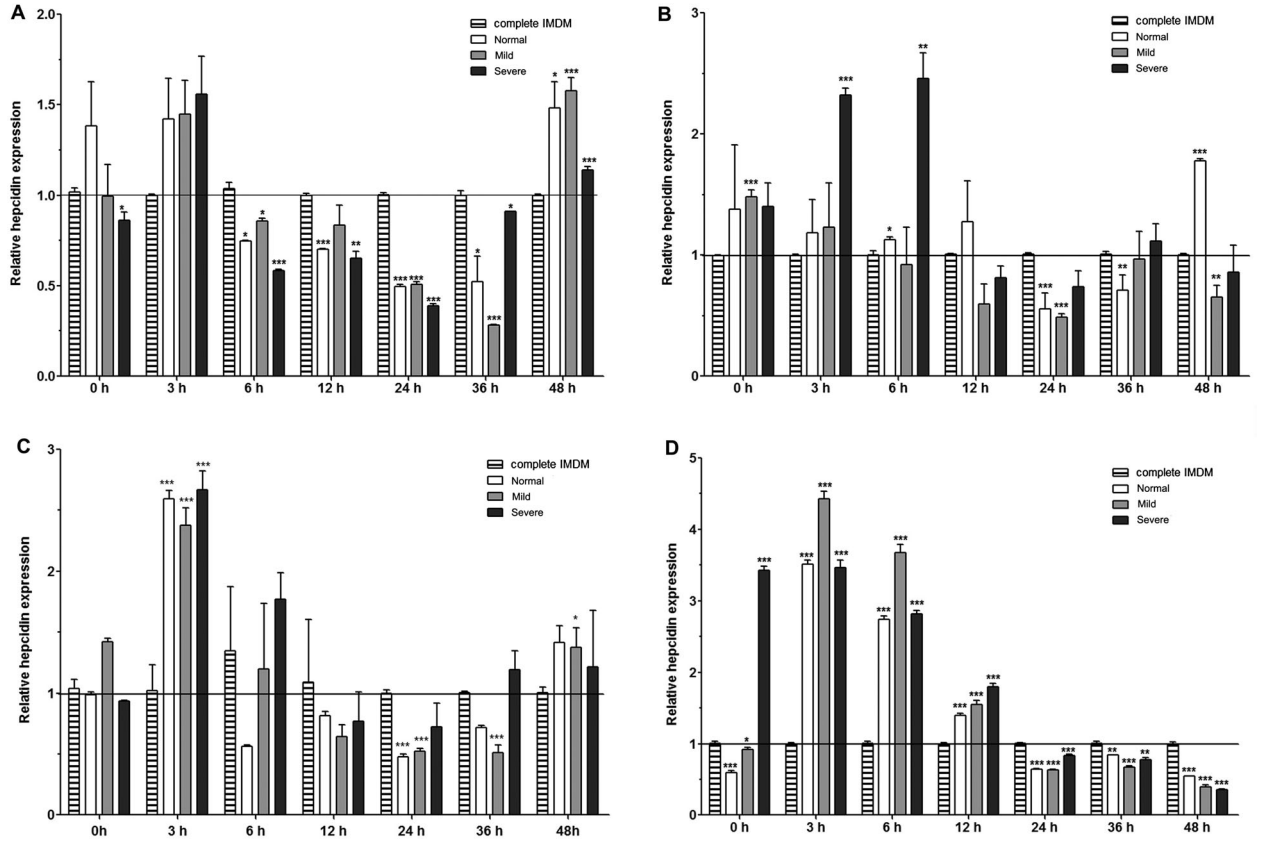

Figure 1. Hepcidin mRNA expression in HepG2 cells after treatment with erythroblast conditioned media. HepG2 cells were treated with (A) Day 3, (B) Day 7, (C) Day 10 and (D) Day 14 conditioned media derived from culture of erythroid cells from normal controls (white bars), mild (gray bars) and severe (black bars) beta0-thalassemia/ Hb E patients. At the times, indicated expression of hepcidin mRNA was determined by qPCR. The relative hepcidin expression was normalized against HepG2 cells treated with complete IMDM media, which had been incubated under the same conditions and for the same time as the erythroid cell culture media (striped bars). Each experiment was undertaken as three independent biological replicates with triplicate qPCR. Error bars represent SEM (*$p < .05$, **$p < .01$, ***$p < .001$ as compared to complete IMDM-treated control cells at the same time point).

Effect of erythroid cell conditioned media on hepcidin expression

To investigate whether media in which erythroid cells had been cultured was able to suppress hepcidin expression in HepG2 cells, HepG2 cells were treated with 2 mL of Days 3, 7, 10 and 14 conditioned media collected during the culture of normal, mild and severe beta0-thalassemia/Hb E erythroblasts. Levels of hepcidin mRNA expression in treated and untreated HepG2 cells were determined using RT-PCR at 0, 3, 6, 12, 24, 36 and 48 hours after treatment. Hepcidin mRNA expression levels were normalized against complete IMDM (IMDM supplemented with 15% heat-inactivated FBS, 15% human AB serum, 2 U/mL rHu erythropoietin, 20 ng/mL rHu stem cell factor) treated HepG2 cells as a control. The results (Figure 1) showed an early (3- to 6-hour posttreatment) upregulation in expression of hepcidin expression (although for some samples, this did not reach statistical significance), followed by suppression of hepcidin expression at around 24 hours posttreatment, for all conditions tested. However, there were some variations between treatments, for example Day 7 conditioned media from the culture of erythroid cells from severe beta0-thalassemia/Hb E patients showed significantly greater upregulation of hepcidin expression at 3 and 6 hours as compared to media from the culture of normal control or mild beta-thalassemia erythroid cells. While conditioned media from all conditions suppressed hepcidin expression at 24 hours, the suppression was more sustained with conditioned media from Day 14 of culture (Figure 1), where hepcidin expression was still suppressed at the last time point examined (48 hours posttreatment).

To confirm the specificity of the suppression of hepcidin expression by erythroid cell conditioned media, conditioned media from the culture of A549 cells (a human lung epithelial cell line) was first evaluated for cytotoxicity to HepG2 cells. Results (Supplemental Figure 2A) showed that A549 conditioned media was not cytotoxic to HepG2 cells for up to 48 hours (the last time point examined). HepG2 cells were again treated with A549 conditioned media in parallel with complete IMDM-treated cells and levels of hepcidin expression determined by RT-PCR. Results (Supplemental Figure 2B) showed that there was an elevation of hepcidin expression as compared to the complete IMDM-treated control cells.

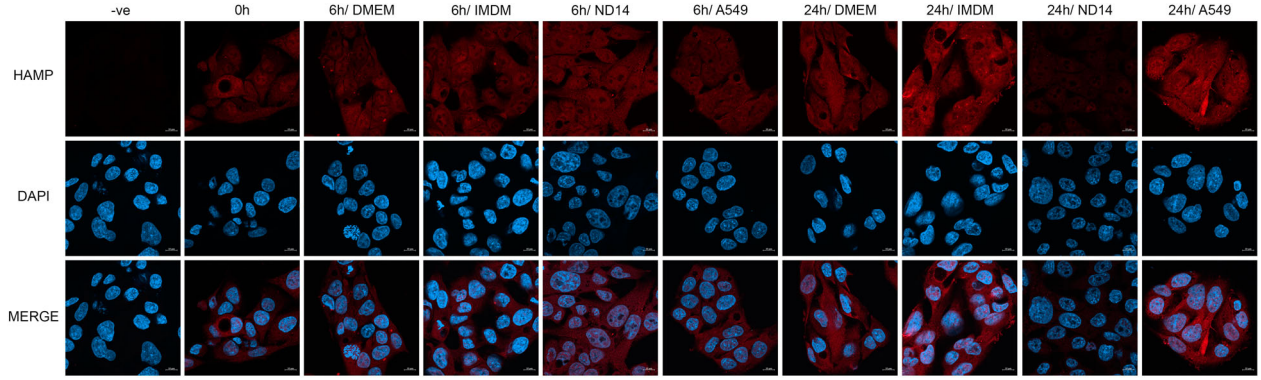

Figure 2. Immunofluorescence assay of hepcidin expression. HepG2 cells grown on coverslips were treated with 10% FBS DMEM, complete IMDM, Day 14 normal control erythroblast conditioned media (ND14) or A549 cells conditioned media (A549) for 6 and 24 hours. Cells were fixed, permeabilized and incubated with a rabbit anti-hepcidin antibody, followed by incubation with an Alexa Flour 647 conjugated donkey anti-rabbit IgG antibody (red signal). The nucleus was stained with DAPI as shown in blue. Negative staining (no primary antibody) of hepcidin in HepG2 cells is shown in the left panel (-ve).

To further confirm the suppression of hepcidin expression by erythroblast conditioned media, HepG2 cells grown on coverslips were treated with DMEM, complete IMDM, conditioned media from A549 cells, or conditioned media from culture of Day 14 erythroblasts from normal controls. Cells were examined for expression of hepcidin at 0, 6 and 24 hours posttreatment by indirect immunofluorescence assay. Results (Figure 2) clearly show the specific inhibition of hepcidin expression by the erythroblast conditioned media at 24 hours posttreatment.

Discussion

The iron regulatory hormone hepcidin plays a major role in systemic iron homeostasis through its interaction with the sole iron exporting protein ferroportin which is expressed predominantly by iron storage cells and on the basolateral membrane of enterocytes [8,25,26]. Hepcidin promotes the internalization and degradation of ferroportin, effectively locking iron inside the cell. Under conditions of low hepcidin expression, ferroportin is expressed on the cell surface, allowing the export of iron from the cell. The predominant demand for iron is erythropoiesis whereby the differentiating erythroid cells require iron for the formation of hemoglobin [12]. Thus, during erythropoiesis, erythroid cells are believed to secrete a factor or factors that suppresses the production of hepcidin in the liver, to allow for iron to be released from iron exporting cells, duodenal enterocytes, hepatocytes, macrophages and spleen cells [8].

The identity of the erythroid factor (or factors) that mediate the suppression of hepcidin expression in the liver remain unclear, although several candidates, including GDF15 [14], TWSG1 [15] and erythroferrone [16], have been proposed to mediate suppression. These erythroid factors are believed to be secreted at different stages of erythroid cell differentiation, with TWSG1 being more highly expressed during early stages of differentiation and GDF15 and erythroferrone being secreted at later stages of differentiation [14–16]. This would accord with our observation that there was some suppression of hepcidin expression by conditioned media at all stages of differentiation examined, suggesting that different stages of erythroid differentiation may secrete different hepcidin expression regulators.

The upregulation of hepcidin expression was consistently observed at an early time point after the addition of conditioned media. This could possibly be occurring as a consequence of the presence of remaining heat-inactivated FBS in the conditioned media, as previous results have shown the increased expression of hepcidin in liver cells at 6 hours as a consequence of serum treatment acting through BMP or a BMP-like molecule that is present in the serum [27]. The suppression of hepcidin expression was consistently seen at 24 hours after addition of erythroid conditioned media, irrespective of the stage of differentiation, and the specificity of the suppression by erythroid conditioned media was confirmed by the use of conditioned media from cultured A459 cells and analysis by both RT-PCR and confocal microscopy. Overall, there was surprisingly little difference in suppression of hepcidin expression as mediated by conditioned media obtained through culture of normal erythroid cells and those obtained from mild and severe beta0-thalassemia/Hb E patients. This would tend to support the proposal of Kautz et al. [16,20] that the low levels of hepcidin seen in beta-thalassemia/Hb E patients is a consequence of the increased mass of differentiating erythroid cells generated by expansion of early-stage erythroblasts as a consequence of ineffective erythropoiesis [19]. Collectively, our results support the proposal that there is no defect in beta0-thalassemia/Hb E erythroid cell signaling for suppression of hepcidin expression and show that erythroid

cell conditioned media treatment of liver cells is a viable model system for investigating the mechanism of erythroid-mediated suppression of liver cell hepcidin expression.

Disclosure of interest

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Thailand Research Fund (BRG5780004 and IRG5780009) and by a Research Chair Grant from the National Science and Technology Development Agency (NSTDA). WS was supported by a Thai Royal Golden Jubilee Ph.D. Research Scholarship (PHD/0101/2553) and JJ was supported by a Thailand Graduate Institute of Science and Technology (TGIST) M.Sc. Scholarship.

ORCID

Janejira Jaratsittisin http://orcid.org/0000-0002-0651-5377
Wannapa Sornjai http://orcid.org/0000-0003-0935-7930
Saovaros Svasti http://orcid.org/0000-0001-8334-9838
Suthat Fucharoen http://orcid.org/0000-0001-6219-9711
Sittiruk Roytrakul http://orcid.org/0000-0003-3696-8390
Duncan R. Smith http://orcid.org/0000-0002-6592-9852

References

[1] MacKenzie EL, Iwasaki K, Tsuji Y. Intracellular iron transport and storage: from molecular mechanisms to health implications. Antioxid Redox Signal. 2008 Jun;10(6):997–1030.
[2] Kaplan J, Ward DM, De Domenico I. The molecular basis of iron overload disorders and iron-linked anemias. Int J Hematol. 2011 Jan;93(1):14–20.
[3] Nemeth E, Ganz T. The role of hepcidin in iron metabolism. Acta Haematol. 2009;122(2–3):78–86.
[4] Valore EV, Ganz T. Posttranslational processing of hepcidin in human hepatocytes is mediated by the prohormone convertase furin. Blood Cells Mol Dis. 2008 Jan-Feb;40(1):132–138.
[5] Ganz T. Systemic iron homeostasis. Physiol Rev. 2013 Oct;93(4):1721–1741.
[6] Nemeth E, Preza GC, Jung CL, et al. The N-terminus of hepcidin is essential for its interaction with ferroportin: structure-function study. Blood. 2006 Jan 01;107(1):328–333.
[7] Peslova G, Petrak J, Kuzelova K, et al. Hepcidin, the hormone of iron metabolism, is bound specifically to α-2-macroglobulin in blood. Blood. 2009;113(24):6225–6236.
[8] Donovan A, Lima CA, Pinkus JL, et al. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. Cell Metab. 2005;1(3):191–200.
[9] Zhang D-L, Senecal T, Ghosh MC, et al. Hepcidin regulates ferroportin expression and intracellular iron homeostasis of erythroblasts. Blood. 2011;118(10):2868–2877.
[10] De Domenico I, Ward DM, Langelier C, et al. The molecular mechanism of hepcidin-mediated ferroportin down-regulation. Mol Biol Cell. 2007 Jul;18(7):2569–2578.
[11] Ganz T, Nemeth E. The hepcidin-ferroportin system as a therapeutic target in anemias and iron overload disorders. Hematology Am Soc Hematol Educ Program. 2011;2011:538–542.
[12] Pak M, Lopez MA, Gabayan V, et al. Suppression of hepcidin during anemia requires erythropoietic activity. Blood. 2006;108(12):3730–3735.
[13] Vokurka M, Krijt J, Sulc K, et al. Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. Physiol Res. 2006;55(6):667–674.
[14] Tanno T, Bhanu NV, Oneal PA, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. Nat Med. 2007 Sep;13(9):1096–1101.
[15] Tanno T, Porayette P, Sripichai O, et al. Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. Blood. 2009 Jul 2;114(1):181–186.
[16] Kautz L, Jung G, Valore EV, et al. Identification of erythroferrone as an erythroid regulator of iron metabolism. Nat Genet. 2014 Jul;46(7):678–684.
[17] Weatherall DJ, Clegg JB. The thalassaemia syndromes. 4th ed. Oxford: Blackwell Science; 2001.
[18] Winichagoon P, Thonglairoam V, Fucharoen S, et al. Severity differences in beta-thalassaemia/haemoglobin E syndromes: implication of genetic factors. Br J Haematol. 1993 Apr;83(4):633–639.
[19] Mathias LA, Fisher TC, Zeng L, et al. Ineffective erythropoiesis in β-thalassemia major is due to apoptosis at the polychromatophilic normoblast stage. Exp Hematol. 2000;28(12):1343–1353.
[20] Kautz L, Jung G, Du X, et al. Erythroferrone contributes to hepcidin suppression and iron overload in a mouse model of beta-thalassemia. Blood. 2015 Oct 22;126(17):2031–2037.
[21] Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics. 2002 Nov;1(11):845–867.
[22] Wannatung T, Lithanatudom P, Leecharoenkiat A, et al. Increased erythropoiesis of beta-thalassaemia/Hb E proerythroblasts is mediated by high basal levels of ERK1/2 activation. Br J Haematol. 2009 Sep;146(5):557–568.
[23] Sripichai O, Makarasara W, Munkongdee T, et al. A scoring system for the classification of beta-thalassemia/Hb E disease severity. Am J Hematol. 2008 Jun;83(6):482–484.
[24] Leecharoenkiat A, Wannatung T, Lithanatudom P, et al. Increased oxidative metabolism is associated with erythroid precursor expansion in beta(0)-thalassaemia/Hb E disease. Blood Cells Mol. Dis. 2011 Oct 15;47(3):143–157.
[25] Abboud S, Haile DJ. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. J Biol Chem. 2000 Jun 30;275(26):19906–19912.
[26] McKie AT, Marciani P, Rolfs A, et al. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell. 2000;5(2):299–309.
[27] Shanmugam NKN, Cherayil BJ. Serum-induced up-regulation of hepcidin expression involves the bone morphogenetic protein signaling pathway. Biochem Biophys Res Commun. 2013;441(2):383–386.