FINAL REPORT

การศึกษาวิเคราะห์มุมมองของ hepcidin-ferroportin ในผู้ป่วยธาลัสซีเมีย Hb E

Analysis of aspects of the hepcidin-ferroportin axis in
β-thalassemia/Hb E disease

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บทคัดย่อ

ประชากรมากกว่าหนึ่งพันล้านคนทั่วโลกได้รับผลกระทบจากภาวะโลหิตจางและภาวะเหล็กเกิน เช่นเดียวกับประชากรไทยจำนวนมาก ที่มียีนเบต้าธาลัสซีเมียหลากหลายรูปแบบ ทำให้ภาวะเหล็กเกินถือเป็นปัญหาสำคัญ แม้ว่าธาตุเหล็กส่วนใหญ่ในร่างกายมีการหมุนเวียนอยู่ในเซลล์เม็ดเลือดแดง-เซลล์แมคโครฟาจ-กระบวนการสร้างเม็ดเลือดแดง-เซลล์เม็ดเลือดแดง แต่การหมุนเวียนของธาตุเหล็กในร่างกายจะถูกควบคุมโดยฮอร์โมนเฮปซิดิน ซึ่งถูกสร้างมาจากเซลล์ตับ ฮอร์โมนเฮปซิดินทำงานโดยการควบคุมการแสดงออกของโปรตีนเฟอร์โรพอร์ติน (ซึ่งเป็นโปรตีนเพียงตัวเดียวในเซลล์ที่ทำหน้าที่ขับธาตุเหล็กออกจากเซลล์) ในขั้นหลังการแปลรหัส โดยจับกับโปรตีนเฟอร์โรพอร์ติน แล้วทำให้เคลื่อนเข้าไปในเซลล์ และเกิดการย่อยสลายของโปรตีนนี้ ทำให้การขับธาตุเหล็กออกจากเซลล์ถูกยับยั้ง นอกจากนี้การควบคุมโปรตีนเฟอร์โรพอร์ตินยังพบในขั้นการถอดรหัสและการแปลรหัส โดยในขั้นการแปลรหัสโปรตีนถูกควบคุมด้วยระดับของธาตุเหล็ก ซึ่งทำงานผ่านทาง iron responsive element (IRE) บนตำแหน่ง 5’ UTR ของเฟอร์โรพอร์ติน mRNA แต่มีการรายงานว่ายีนนี้ในเซลล์ตัวอ่อนของเม็ดเลือดแดง มีอีกหนึ่ง upstream promoter ที่สามารถสร้างเฟอร์โรพอร์ติน mRNA ชนิดที่ไม่มี IRE ได้ และมีความเชื่อมั่นว่าการทำงานร่วมกันของ mRNA ทั้งสองชนิดนี้มีความสำคัญอย่างยิ่งในระหว่างกระบวนการสร้างเม็ดเลือดแดง แต่อย่างไรก็ตาม ยังไม่มีการศึกษาเรื่องนี้ในผู้ป่วยเบต้าธาลัสซีเมีย/ฮีโมลโกลบินอี ซึ่งมีภาวะการสร้างเม็ดเลือดแดงที่ไม่มีประสิทธิภาพ จากการศึกษาของเราพบว่าในระหว่างกระบวนการสร้างเม็ดเลือดแดงของผู้ป่วยเบต้าธาลัสซีเมีย/ฮีโมลโกลบินอี ยีนเฟอร์โรพอร์ตินมีการแสดงออกที่ผิดปกติไป และไม่สามารถเพิ่มการแสดงออกแม้อยู่ภายใต้ภาวะเหล็กเกิน ซึ่งอาจนำไปสู่การเกิดภาวะการสร้างเม็ดเลือดแดงที่ไม่มีประสิทธิภาพ นอกจากนี้การศึกษาของเราค้นพบเป็นครั้งแรกว่า เซลล์ตัวอ่อนของเม็ดเลือดแดงที่กำลังพัฒนาสามารถสร้างฮอร์โมนเฮปซิดินได้ และด้วยระบบที่พัฒนาขึ้นมาใหม่ เราสามารถระบุโปรตีนควบคุมตัวใหม่ที่มีขนาดต่ำกว่า 3 กิโลดาลตัน ซึ่งทำหน้าที่ควบคุมการแสดงออกของฮอร์โมนเฮปซิดินในเซลล์ตับ นอกจากนี้เรายังได้ตรวจสอบถึงปัจจัยที่มีผลต่อการคงอยู่ในระดับสูงของยีนธาลัสซีเมีย ในกลุ่มประชากรเอเชียตะวันออกเฉียงใต้ คณะผู้วิจัยได้แสดงให้เห็นว่าผู้เป็นพาหะธาลัสซีเมียสามารถต้านทานต่อการติดเชื้อไวรัสเดงกี่ในเซลล์ตัวอ่อนของเม็ดเลือดแดงได้ ซึ่งอาจเกิดจากความผิดปกติของกระบวนการสร้างไรโบโซมในเซลล์ตัวอ่อนเม็ดเลือดแดงของผู้ที่เป็นพาหะธาลัสซีเมีย ที่ทางเราได้ค้นพบเป็นครั้งแรกเช่นกัน โดยรวมแล้วจากการศึกษาของเราได้แสดงให้เห็นถึงหลายแง่มุมใหม่ของธาลัสซีเมีย

คำสำคัญ: ธาลัสซีเมีย, ธาตุเหล็ก, เฟอร์โรพอร์ติน, เซลล์เม็ดเลือดแดงตัวอ่อน, ฮอร์โมนเฮปซิดิน, ภาวะการสร้างเม็ดเลือดแดงที่ไม่มีประสิทธิภาพ

ABSTRACT

Iron deficiency anemia and iron over load conditions affect more than one billion people worldwide. In Thailand with its high number of people living with various forms of β-thalassemia, iron overload disease is a significant concern. Although the main iron cycle is red blood cell - macrophage -erythropoiesis - red blood cell, the cycle is predominantly controlled by a peptide hormone, hepcidin, produced by hepatocytes. Hepcidin post-translationally regulates a protein called ferroportin (which is the sole iron exporting protein expressed by cells) by binding to it and promoting its internalization and subsequent degradation, halting export of iron from the cell. Ferroportin is additionally controlled at the levels of transcription and translation. Translational regulation is mediated by iron status acting through the presence of an iron responsive element (IRE) in the 5'-UTR of the ferroportin mRNA. The existence of a ferroportin mRNA without the IRE produced by a more upstream promoter has been reported in erythroid cells, and it is believed that co-ordinate regulation of these two messages during erythropoiesis is essential. However, this has not been previously investigated in ineffective β-thalassemia/Hb E erythropoiesis. Our study has shown that ferroportin gene expression is dsyregulated during β-thalassemia/Hb E erythropoiesis and that there is a failure to up-regulate ferroportin expression during conditions of iron overload, possibly driving ineffective erythropoesis. Our studies additionally showed for the first time that hepcidin is produced by developing erythroblasts. More importantly using a newly developed system we have provisionally identified a novel sub-3kda regulator of hepcidin expression in liver cells. In addition we started to address the factors keeping high levels of thalassemia genes in the Southeast Asian population, and we demonstrated that trait carrier status reduces the susceptibility of erythroblasts to infection with Dengue virus. This is possibly mediated by dysregulated ribosome biogenesis, which we documented for the first time in thalassemia trait erythroblasts. Combined, these studies have shed new light on a number of aspects of thalassemia.

Key words: β-thalassemia; iron; ferroportin; erythroblasts; hepcidin; ineffective erythropoiesis

Executive Summary

Introduction

Iron overload and iron deficiency anemia's affect more than a billion people worldwide. Iron homeostasis involves the regulation of cells that export iron into the plasma and cells that utilize or store iron. The cellular iron balance in humans is primarily mediated by the hepcidin-ferroportin axis. Ferroportin is the sole cellular iron export protein, and it expression is regulated transcriptionally, post-transcriptionally and post-translationally. Hepcidin, a hormone produced by liver cells post-translationally regulates ferroportin expression on cells by binding with ferroportin and promoting its internalization by endocytosis and subsequent degradation by lysosomes. Classically it is known that translational regulation of ferroportin is mediated by the presence of iron responsive elements (IREs) in the 5'-untranslated region of the ferroportin mRNA and the action of iron responsive proteins (IRP1 and IRP2). Under conditions of low iron, IRPs bind to the ferroportin IRE and repress translation of ferroportin. This has the effect of retaining intracellular iron when iron availability is limited.

Studies have shown that erythroid precursor cells express ferroportin, but that expression is controlled by both an IRE containing mRNA (FPN1A) and a non-IRE containing mRNA (FPN1B), with the non-IRE containing mRNA (FPN1B) being generated through alternative splicing of the FPN gene, although the mature protein generated by either transcript is the same. Studies have suggested that early in erythroid cell differentiation FPN1B transcripts predominate to allow export of iron from the cells during early differentiation independent of IRP/IRE repression, but that later in differentiation when heme production begins FPN1B expression declines and FPN1A predominates, again placing the cells under iron status control.

Our earlier studies have shown that erythroid precursor cells from β-thalassemia/HbE patients are insensitive to a number of external stimuli, including EPO and serum deprivation. Our hypothesis is that erythroid precursor cells are insensitive to the signals that should promote the co-ordinate regulation of FPN1A and FPN1B, and this would lead to loss of iron from erythroid precursors when the iron should be available for heme production. This hypothesis is supported by data that shows that erythroid precursor cells may undergo apoptosis at the polychromatophilic erythroblast stage as a consequence of iron deficit at this point in differentiation. Ferroportin can be post-transcriptionally down regulated by hepcidin, a hormone produced in liver cells in response to as yet incompletely characterized messages from the erythroid cell mass. In β-thalassemia patients hepcidin is expressed at extremely low levels, and this is thought to contribute to the iron overload status of both transfused and non-transfused β-thalassemia patients. However, to date the effects of hepcidin on thalassemic erythroid progenitor cells has not been characterized. However, if our hypothesis is correct, treatment of thalassemic erythroid progenitor cells with hepcidin at appropriate time points should decrease ineffective erythropoiesis.

Lastly, as noted, the nature of the signal from the erythroid mass to the liver cells is not fully characterized, and this study seeks to address that point. Currently it is believed that the mechanism by which erythropoiesis signals hepatocytes to reduce hepcidin expression is possibly mediated by growth differentiation factor 15 (GDF-15; which is extremely elevated in β-thalassemia patients) and the twisted gastrulation protein (TWSG1) both of which have been implicated by studies, but other studies have suggested that other circulating factor may play the decisive role. To date, studies investigating the link between erythropoiesis and hepcidin regulation by liver cells have used control and patient serum. Serum is an extremely complex solution and contains the largest proteome in humans. This study intended to investigate the link between erythropoiesis and hepcidin suppression using conditioned medium from cultured erythroid cells from β-thalassemia/Hb E patients with different severities to provide a more controlled medium suitable for further analysis. Combined this study seeks to undertake a comprehensive analysis of the hepcidin-ferroportin axis in β-thalassemia/Hb E, to determine points of therapeutic intervention.

Results and Discussion

Thalassemia and the hepcidin-ferroportin axis: Ferroportin

Ferroportin is the sole iron exporting protein expressed by cells. While erythroid cells are predominantly iron importing cells, with the iron required for hemoglobin synthesis, the cells still have to be capable of regulating cellular iron levels to avoid iron overload which could have significant consequences for the cell. We therefore initially investigated expression of this protein in developing erythroid cells from thalassemia patients of different severities as compared to normal control cells.

Ferroportin (FPN1) also known as Iron-regulated transporter 1 or Solute carrier family 40 member 1 is encoded by the *SLC40A1* gene [Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000]. This gene is composed of 8 exons that give rise to a 571 amino acid protein of approximately 62 kDa [Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000]. Notably, erythroid cells contain two major FPN1 transcripts, an iron responsive element (IRE)-containing transcript termed FPN1a or *variant I* and non-IRE-containing transcripts FPN1b or *variant II*. Two different forms of *variant II* (A and B) have been reported [Cianetti et al., 2005]. However the protein encoded by all transcripts is identical (Figure 1). For this reason, this study sought to determine the expression profile of both *variant I* and *variant II* FPN1 mRNA during erythropoiesis of normal and β^0-thalassemia/Hb E erythroblasts.

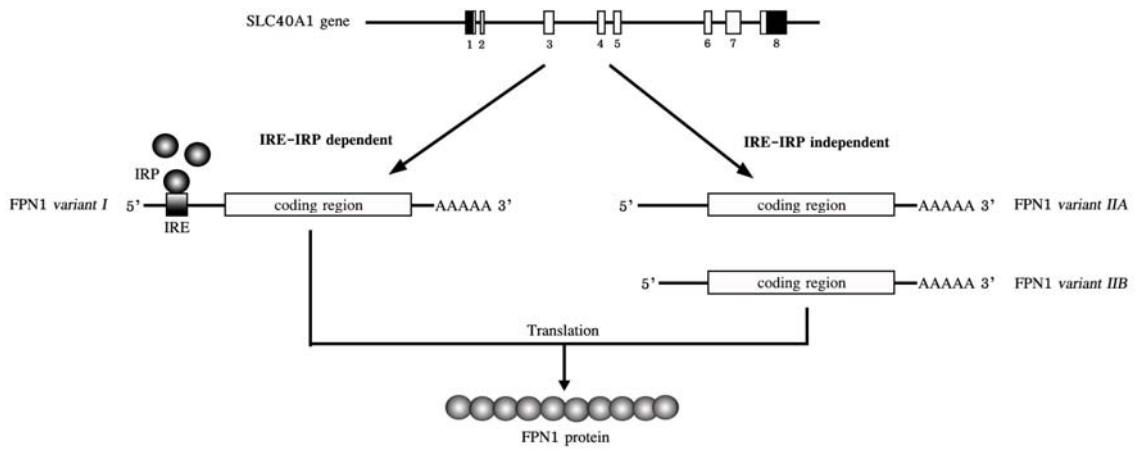

Figure 1. Organization and transcription/translation of the ferroportin gene

Normal control erythroblasts showed levels of total FPN1 and FPN *variant I* mRNAs that were markedly higher on days 7 and 14 as compared to day 10, consistent with previous reports [Cianetti et al., 2010; Cianetti et al., 2005]. The reduction in FPN1 and FPN *variant I* expression on day 10 of culture correlates with the increased demand for iron with the major period of hemoglobin synthesis [Wojda et al., 2002], while increased expression on days 7 and 14 would serve to allow the export of potentially toxic iron from these cells when there is no increased requirement for iron. While erythroid cells from mild β^0-thalassemia/Hb E patients were consistent with this pattern of expression, the reduction in total FPN1 on day 10 was significantly less than for normal control erythroblasts, with cells from mild β^0-thalassemia/Hb E patients showing only a small decrease in expression from the day seven levels. This is consistent with cells from mild β^0-thalassemia/Hb E patients showing dysregulation of ferroportin that is intermediate between cells from normal controls and severe β^0-thalassemia/Hb E patients. In contrast the pattern of expression of total FPN1 in erythroid cells from severe β^0-thalassemia/Hb E patients was one of a decrease in expression throughout the period examined, with no increase in FPN1 expression on day 14 as was seen with cells from both normal controls and from mild β^0-thalassemia/Hb E patients. Markedly however, differences in expression of both total FPN1 and the iron regulated *variant I* mRNA in β^0-thalassemia/Hb E erythroblasts as compared to normal control erythroblasts occur only from day 10, suggesting that it is the major period of globin synthesis that results in the disordered expression of the iron regulated *variant I* mRNA, which is reflected in total FPN1 mRNA expression.

It is proposed that expression of the *variant II* messages predominantly occurs during the intermediate stage of erythroid differentiation, although our results showed relatively robust expression of *variant IIA* at all stages examined. *Variant IIB* expression was markedly reduced in comparison to *variant IIA*. Expression of *variant IIA* was higher in erythroid cells from β^0-thalassemia/Hb E patients as compared to normal controls on days 7 and 10, while *variant IIB* was significantly increased in erythroid cells from β^0-thalassemia Hb E patients as compared to normal controls on all days examined. This suggests that thalassemic erythroblasts, particularly in severe cases, may compensate for reduced *variant I* mRNA expression by increasing *variant II* expression with the increased early expression (day 7) suggesting that this is initiated as a consequence of the onset globin synthesis.

FPN1 protein as assessed by western blotting was found to be not significantly different, suggesting that the increased *variant II* expression compensated for the markedly reduced *variant I* expression seen in severe cases. As the *variant II* messages do not contain an IRE, these cells will be less sensitive to iron overloading, which could result in the increased labile iron pools seen in β^0-thalassemia erythroblasts in some studies [Prus and Fibach, 2008]. The cellular consequences of iron overload can include increased cell death, consistent with the known pathophysiology of β^0-thalassemia [Mathias et al., 2000].

Thalassemia and the hepcidin-ferroportin axis: Hepcidin

The 25 amino acid, 2789.8Da peptide hormone hepcidin (also known as liver-expressed antimicrobial peptide 1) is the master regulatory control peptide for iron homeostasis [Nemeth and Ganz, 2009]. 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 mature 25 amino acid mature peptide hormone [Valore and Ganz, 2008], which possesses a β-hairpin structure containing four disulfide bonds [Ganz, 2013]. Six N-terminal amino acids are highly conserved and essential for hepcidin activity [Nemeth et al., 2006]. 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 α_2-macroglobulin [Peslova et al., 2009].

Hepcidin acts through its interaction with its receptor protein, ferroportin, which is the only known iron exporter protein located on plasma membrane of professional iron exporting cells such as duodenal enterocytes, hepatocytes, macrophages, spleen cells [Donovan et al., 2005] and erythroid precursor cells [Zhang et al., 2011]. 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 [De Domenico et al., 2007]. 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 regulating iron recycling from senescent erythrocytes in the spleen [Ganz and Nemeth, 2011]. Apart from regulation by the plasma iron concentration, hepcidin production is also directly regulated by erythropoietic activity in the bone marrow [Pak et al., 2006]. 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 [Vokurka et al., 2006], and a number of mediators of this suppression have been proposed, including growth differentiation factor 15 (GDF15) [Tanno et al., 2007], twisted gastrulation protein (TWSG1) [Tanno et al., 2009] and erythroferrone [Kautz et al., 2014a].

However, previous studies investigating erythroid factors that mediate hepcidin suppression were based on analysis of plasma of β-thalassemia patients [Kautz et al., 2014b; Tanno et al., 2007; Tanno et al., 2009], and plasma is known as a complex fluid whose composition is influenced by many cells [Anderson and Anderson, 2002]. To address this, we sought to develop an experimental system that would allow investigation of specifically erythroid secreted factors that mediate hepcidin expression.

HepG2 cells were evaluated as a model system to investigate erythroid mediated suppression of hepcidin expression. A conditioned media approach was utilized, in which erythroid cells were cultured in normal growth media, and the media then used to treat HeG2 cells and the expression of hepcidin determined. It was shown that the conditioned media was not toxic to HepG2 cells. Conditioned media from both thalassemia patients of different severity, as well as normal controls were investigated, and it was shown that all conditioned media resulted in the suppression of hepcidin expression at 24 hours post treatment. Interestingly, a strong upregulation of hepcidin was observed at 6 hours after treatment. A preliminary characterization using size separation chromatography showed that the factor suppressing hepcidin expression has a molecular weight of less than 3kDa, and is thus not one of the previously identified regulators of hepcidin expression. Hepcidin itself was rules out, although it was also shown that erythroid cells can express hepcidin.

The thalassemia trait: protection against dengue virus

Thalassemia is prevalent in Thailand, and the presentation of thalassemia here is complex due to the circulation of a number of different thalassemias and hemoglobinopathies in the population. These genes are often co-inherited leading to the complex pattern of thalassemia seen in Thailand. One significant question that remains to be answered is why are these thalassemia traits maintained at such high levels?

Erythroid cells have been well characterized as a target for mosquito borne protozoan parasite *Plasmodium* spp., which are responsible for the disease malaria [Garcia, 2010] and the protective effects of hemoglobin variants or globin gene deletions has been well established [Taylor et al., 2012]. For example heterozygous sickle cell traits have been well characterized as providing a protective advantage against malaria in endemic areas [Aidoo et al., 2002; Allison, 1954; Piel et al., 2010; Williams et al., 2005]. Similarly, α+-thalassemia traits have been proposed as another determinant that may provide protection against severe malaria [Enevold et al., 2007; Mockenhaupt et al., 2004; Wambua et al., 2006]. These studies support the malaria hypothesis which proposes that it is the selective pressure provided by the malaria parasite that maintains the sickle cell and thalassemia traits in populations [Clegg and Weatherall, 1999]. In some areas of the world, and particularly in Southeast Asia, thalassemia traits are found in the population at extremely high levels [Fucharoen and Winichagoon, 1992]. In parts of Thailand the hemoglobin variant Hb E is found in up to 50% of the population and other traits show similarly high penetrance [Fucharoen and Winichagoon, 1992]. It is possible therefore that other mechanisms besides malaria are providing selective pressure on maintaining these traits in the population. We sought to determine whether erythroid precursor cells were susceptible to DENV infection, and whether thalassemia traits resulted in any modulation of DENV infectivity.

Our study confirmed the susceptibility of erythroid progenitor cells to DENV infection using a high passage laboratory strain of DENV and additionally showed that erythroid committed precursor cells are

also susceptible to DENV infection with the same strain. The study showed that DENV infection of these cells was specific, as we failed to detect infection of these cells with another mosquito borne virus, namely CHIKV an *Alphavirus* of the family *Togaviridae*. Interestingly, the data showed that maximum susceptibility was observed on day 7 of differentiation, in which the majority of cells are predominantly basophilic erythroblasts. HSCs cultured for 4 days (erythroid progenitor) and 10 days (polychromatophilic erythroblasts and orthochromatic erythroblast) showed much lower rates of infection, suggesting that infection may be mediated by a receptor protein that is transiently expressed around the basophilic erythroblast stage. Infection of erythroid committed precursor cells only occurs through direct infection, and we found no evidence of antibody dependent enhancement of infection as occurs with monocytic cells [Halstead et al., 1980] which corresponds to earlier results that showed that DENV-4 infection of bone marrow progenitor cells was not altered by the presence of antibodies [Nakao et al., 1989].

Surprisingly, we found a significant reduction in the susceptibility of erythroid committed precursor cells from carriers of thalassemia traits as compared to normal control cells. The protective effects of various globin gene expression hemoglobinopathies and gene deletions in respect of the protozoan parasite Plasmodium have been well documented, but this is the first report of a similar effect in respect of a flaviviral infection. The mechanism of protection in thalassemia carriers remains unclear. In our system both α- and β-globin chains are expressed at low levels on day 7 and show a marked increase in expression level by day 10 [Leecharoenkiat et al., 2011a]. This coincides with both the maximum susceptibility of these cells, as well as the maximum protection seen in trait erythroid progenitor cells which were infected on day 7 and analyzed 48 hours later (at day 9). Although there is no definitive evidence that thalassemia traits result in a significantly disordered cell processes, it seems likely that even minor imbalances or disorders in globin synthesis can have widespread cellular consequences.

Interestingly, low passage viruses showed only scattered cells with NS1 positive staining indicative of infection but the low passage isolates induced extremely high levels of cell death as compared to the laboratory adapted virus. Our data suggested that the cell death is induced through activation of capases 8 mediated apoptosis in bystander (uninfected) cells. In this case, although only very few cells maybe infected, bystander apoptosis may be a significant factor in bone marrow suppression.

Bystander apoptosis would serve to dampen infection through removing cells that could otherwise be productively infected adding to the viral load. It is interesting that committed erythroid precursor cells from thalassemia trait carriers both showed a reduced susceptibility to the laboratory adapted virus, as well as markedly increased levels of cell death with low passage isolates as compared to normal control committed erythroid precursor cells. Both of these observations would support that the presence of a thalassemia trait may protect in a small way against severe dengue disease.

The thalassemia trait: dysregulation of ribosome biosynthesis

As noted above, the increased protection against dengue infection seen with in erythroid cells of thalassemia trait carriers may imply some subtle global cellular deficit. Ribosome biogenesis is the process of synthesis of the cellular ribosomes which mediate protein translation. Ribosome biogenesis involves more than 170 accessory factors and involves a number of transcriptional and post-transcriptional events [Coute et al., 2006]. Mature ribosomes consist of four different ribosomal RNA (rRNA) molecules together with some 80 different ribosomal proteins, and the rRNA molecules undergo extensive co- and post-transciptional modification including endo and exo-nuclease cleavage, as well as specific 2'-O-methylation and pseudouridylation [Baxter-Roshek et al., 2007]. 2'-O-methylation is regulated by box C/D snoRNAs (small nucleolar RNAs) in association with fibrillarin, while box H/ACA snoRNAs in association with dyskerin regulate pseudouridylation [Henras et al., 2008]. These biochemical modifications could finely tune the catalytic ribozyme activity of the rRNAs which directly control protein synthesis [Baxter-Roshek et al., 2007]. Indeed, evidence is emerging that these post-transcriptional modifications serve to modulate ribosomal assembly and translational capacity [Xue and Barna, 2012]. Defects in ribosome biogenesis have been associated with several inherited bone marrow failure syndromes including Diamond-Blackfan anemia [Narla and Ebert, 2010] which has been shown to result from mutations in a number of ribosomal protein genes [Vlachos et al., 2008]. Given that β-thalassemia is the most common anemia worldwide, we sought to determine if there was evidence of altered ribosome biogenesis in β-thalassemia and additionally looked at hemoglobin E trait.

Two independent cohorts of β-thalassemia trait carriers, showed hypermethylation of 28S rRNA at position 4506. Evidence in both cohorts supports increased fibrillarin expression, although this did not reach significance in the erythroid cell experiment, possibly due to the small sample size. Additionally increased fibrillirin expression, together with increased expression of ancillary factors was observed in HbE trait carriers, suggesting that the alterations of ribosome biogenesis may be widespread in beta-hemoglobinopathies. The data showing evidence of altered 2'-O-methylation of rRNA in 28S rRNA and increased levels of fibrillarin expression in β-thalassemia trait carriers together provide the first evidence of ribosome biogenesis dysfunction in these trait carriers. Defects in ribosome biogenesis have been associated with cancer and aging [Lempiainen and Shore, 2009] as well as with inherited bone marrow failure syndromes [Narla and Ebert, 2010] which have some parallels with β-thalassemia. Our results show that alteration in rRNA ribose methylation patterns could contribute to translational defects in thalassemia.

Details of research

Thalassemia and the hepcidin-ferroportin axis: Ferroportin

To investigate the expression of FPN1 during normal and ineffective erythropoiesis, CD34+ HSCs were isolated from normal control, mild and severe β^0-thalassemia/Hb E patients. The cells were grown under conditions driving erythroid lineage differentiation. Under these conditions, on day 7 of culture the cell population is predominantly pronormoblast and basophilic normoblast and the cells mature to later stages under continued culture [Leecharoenkiat et al., 2011b]. In β^0-thalassemia/Hb E, ineffective erythropoiesis results from excess α-globin chain accumulation which starts to occur when cells initiate globin synthesis at the basophilic normoblast stage (starting around day 7 of culture). FPN1 mRNA expression was therefore examined in erythroid cells from both normal controls and β^0-thalassemia/Hb E patients on days 7, 10 and 14 of culture. Total FPN1 expression was initially determined by real time quantitative PCR. The result showed that total FPN1 mRNA levels varied during normal erythroid differentiation, with highest expression on days 7 and 14 of culture, and decreased expression was observed on day 10 of culture. A similar trend was observed in erythroblasts from mild β^0-thalassemia/Hb E patients, although the expression levels of day 10 total FPN1 were significantly higher than normal controls. Interestingly, the total FPN1 expression of severe β^0-thalassemia/Hb E erythroblasts was relatively constant during differentiation and was significantly down-regulated on day 14 as compared to erythroblasts from both normal controls and mild β^0-thalassemia/Hb E patients.

Erythroid cells contain 2 FPN1 transcript forms which are translated to the identical FPN1 proteins (Figure 1). To clarify the dysregulation of FPN1 expression, each variant mRNA was specifically examined during cell differentiation. Expression of FPN1 *variant I* for both erythroblasts from normal controls and mild β^0-thalassemia/Hb E patients showed the same alteration in expression as seen for total FPN1, with reduced expression in day 10 as compared to both days 7 and 14. As seen for total FPN1 expression, day 10 erythroblasts from mild β^0-thalassemia/Hb E patients showed significantly higher expression than erythroblasts from normal controls, and additionally as compared to erythroblasts from severe β^0-thalassemia/Hb E patients. *Variant I* expression in erythroblasts from severe β^0-thalassemia/Hb E patients showed reduced expression on day 10 as compared to days 7 and 14, but the level of expression was significantly reduced on days 10 and 14 as compared to erythroblasts from normal controls and mild β^0-thalassemia/Hb E patients (Figure 2).

Alternative splicing generates two *variant II* mRNAs, *variant IIA* and *variant IIB*. To distinguish these transcripts, semi-quantitative PCR was used. The PCR products generated were 503 bp (*variant IIA*) and 309 bp (*variant IIB*), and expression was normalized against β-actin (Figure 2). Surprisingly, expression of *variant IIA* (Figure 2) was up regulated in both mild and severe β^0-thalassemia/Hb E erythroblasts as compared to normal control erythroblasts on days 7 and 10 (Figure 2D). Expression of

variant IIB (Figure 2) was up regulated in erythroblasts from mild and severe β^0-thalassemia/Hb E patients as compared to the cells from normal controls for all days examined.

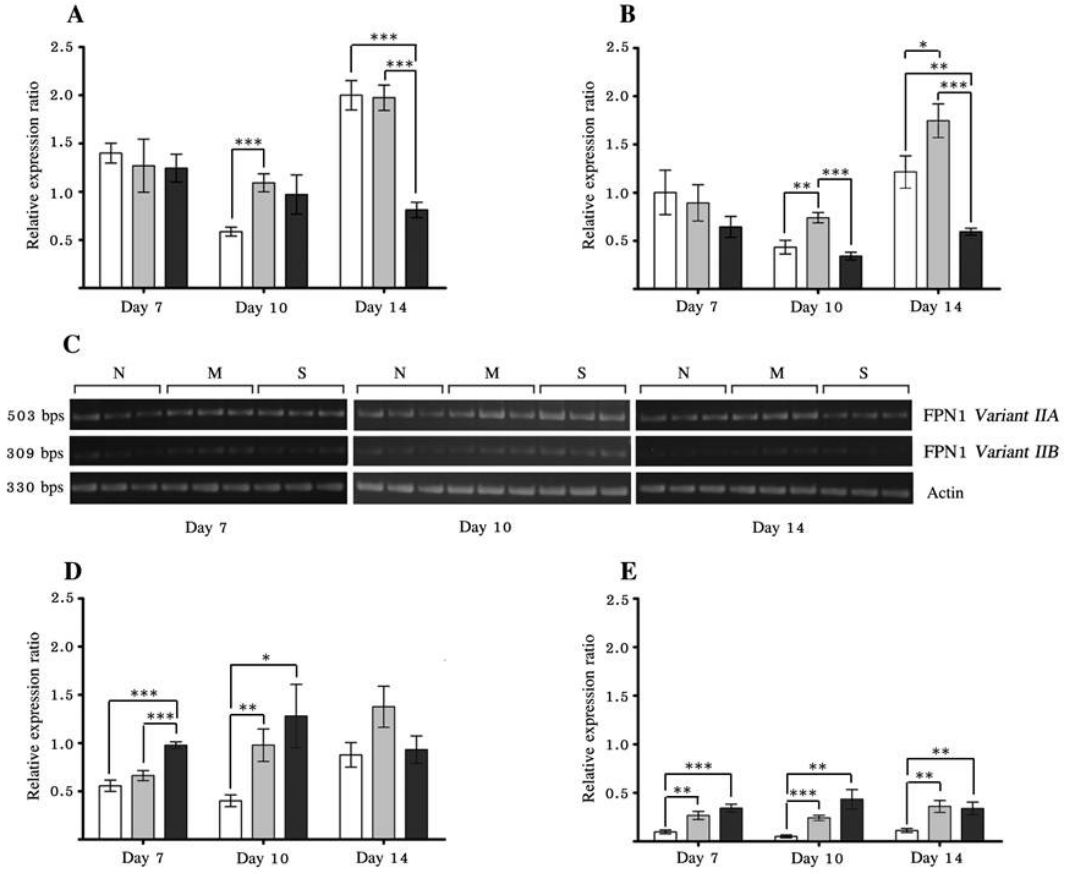

Figure 2. Ferroportin 1 mRNA expression of erythroid cells

The expression of total ferroportin 1 (FPN1) mRNA expression (A), FPN1 *variant I* (B) was assessed by quantitative PCR on day 7, 10 and 14 erythroid cells from normal controls (white bar), mild (light gray bar) and severe β^0-thalassemia/Hb E (dark gray bar) patients. The expression of *variant IIA* and *variant IIB* (C) were assessed by duplicated semi-quantitative PCR and their PCR products were separated on 2% agarose gel (N); normal control, (M) mild β^0-thalassemia/Hb E and (S) severe β^0-thalassemia/Hb E. The expression of *variant IIA* (D) and *variant IIB* (E) were normalized against β-actin and data is shown as relative expression ratio. Error bars represent standard error of the mean (SEM) (*; $p < 0.05$, **; $p < 0.01$ and ***; $p < 0.001$).

FPN1 protein expression in erythroid cells

The expression of FPN1 protein was determined in erythroblasts from normal controls and mild and severe β^0-thalassemia/Hb E patients on days 7, 10 and 14 of culture by western blotting, with β-actin expression as a control. Expression was normalized against β-actin. The results show that no significant difference was observed in the expression levels of FPN1 protein as compared between erythroblasts from normal controls and β^0-thalassemia/Hb E patients.

Transferrin receptor 1 and ferritin heavy chain mRNA expression in erythroid cells

To determine whether the observed FPN1 dysregulation was a systemic defect in iron regulation, the expression of two further genes, transferrin receptor 1 (TFR1) which is involved in cellular iron uptake and ferritin heavy chain (FtH) which is involved in cellular iron storage were determined using real time quantitative PCR. The results showed no significant differences in either TFR1 or FtH expression levels, with expression of both genes being relatively constant during differentiation and between erythroblasts from normal controls and from β^0-thalassemia/Hb E patients, although we noted large inter-sample variation existed.

Iron levels and ferroportin expression

To determine labile iron concentrations in maturing erythroblasts, day 10 and 14 erythroblasts were examined using a colorimetric ferrozine-based labile iron assay. Results showed no significant difference in labile iron concentration between normal controls and severe β-thalassemia/Hb E erythroblasts (Figure 3). We next incubated normal and severe β-thalassemia/Hb E erythroblasts in normal culture medium supplemented with ferric ammonium sulfate (FAS) and observed that incubation with 1mM FAS significantly increased labile iron concentrations in both normal control and severe β -thalassemia/Hb E erythroblasts (Figure 3). This increase in cellular labile iron was not associated with increased cell death (Figure 3). Cells were then examined for expression of total FPN1 mRNA (Figure 5E), the iron regulated *Variant I* mRNA (Figure 3), as well as expression of the non-iron regulated *variant IIA* (Figure 3) and *variant IIB* transcripts (Figure 3). The results clearly show that while normal control erythroblasts significantly up-regulated total FPN mRNA in response to increased iron concentrations in the media, erythroblasts from severe β-thalassemia/Hb E erythroblasts showed no increase in total FPN mRNA expression (Figure 3). Examination of the individual variants (Figure 3) showed the increase of FPN mRNA in normal control erythroblasts was due to an increase in expression of *variant I* expression, which did not occur in the severe β -thalassemia/Hb E erythroblasts.

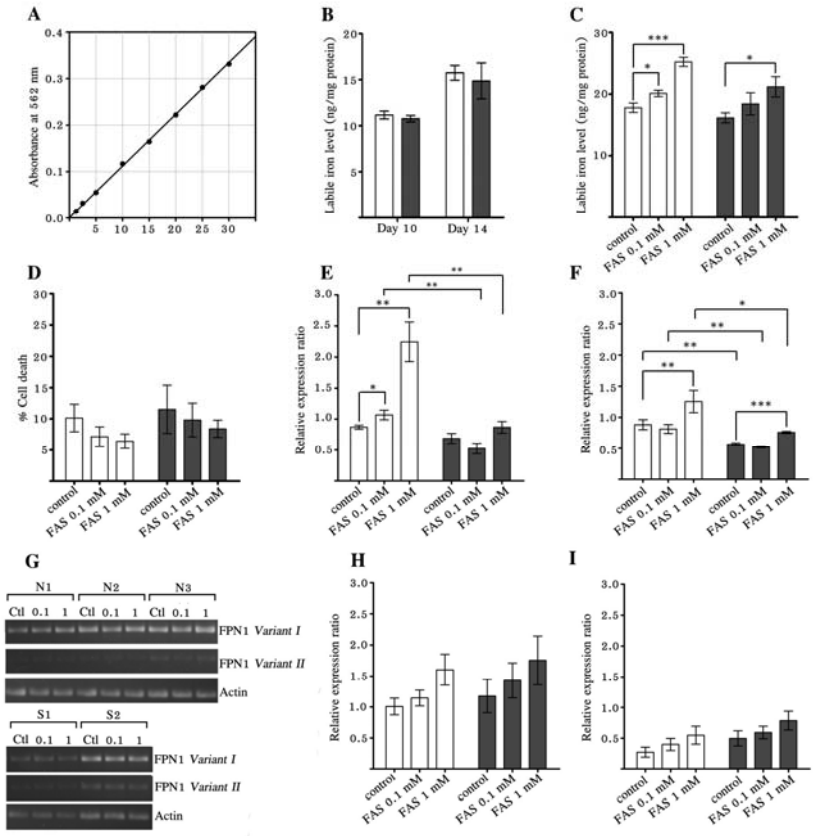

Figure 3. The effect of increased iron on ferroportin 1 mRNA expression in erythroid cells

(A) Labile iron levels were determined by using a colorimetric-ferrozine based assay with $FeCl_3$ as a standard. The formation of Fe^{2+}-ferrozine complex was measured at 562 nm and absorbance was plotted as a linear graph with a correlation coefficient of 0.999 (A). (B) An independent cohort of erythroblasts from 5 normal controls (white bars) and 5 severe β^0-thalassemia/Hb E patients (gray bars) were grown and labile iron levels were measured on days 10 and day 14. (C) To modulate cellular labile iron levels day 13 erythroid cells of 3 normal controls (white bars) and 2 severe β^0-thalassemia/Hb E patients (gray bars) were incubated with ferric ammonium sulfate (FAS). (D) Cell death of cells treated with FAS for 24 hours was evaluated through trypan blue exclusion assays. The effect of increased ambient on the expression of total ferroportin 1 (FPN1) mRNA (E), FPN1 *variant I* (F) was assessed by quantitative RT-PCR and data is shown after normalization against β-actin. The expression of *variant IIA* and *variant IIB* (G) of untreated cells (ctl), FAS 0.1 mM (0.1) and FAS 1 mM (1) was assessed by duplicated semi-quantitative PCR for normal controls (N1, N2, N3) and severe β^0-thalassemia/Hb E erythroid cells (S1, S2). The expression of *variant IIA* (H) and *variant IIB* (I) mRNAs were normalized against β-actin and data is shown as a relative expression ratio. Error bars represent standard error of the mean (SEM) (*; $p < 0.05$, **; $p < 0.01$ and ***; $p < 0.001$).

Thalassemia and the hepcidin-ferroportin axis: hepcidin

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 [Wannatung et al., 2009]. 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 β°-thalassemia/Hb E patients for 24 hours, after which cell viability was assessed by the trypan blue exclusion test. Results showed no significant cytotoxicity of erythroblast conditioned media towards HepG2 cells.

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 day 3, 7, 10, 14 conditioned media collected during the culture of normal, mild and severe β^{0}-thalassemia/Hb E erythroblasts. Levels of hepcidin mRNA expression in treated and untreated HepG2 cells were determined using real-time PCR at 0, 3, 6, 12, 24, 36 and 48 h after treatment. Hepcidin mRNA expression levels were normalized against complete IMDM treated HepG2 cells as a control. The results (Figure 4) showed a broadly consistent pattern, with an early (3 to 6 hours post treatment) up-regulation in expression of hepcidin, followed by suppression of hepcidin expression at around 24 hrs post-treatment for all treatments. There were some variations between treatments with conditioned media from culture of erythroid cells from normal controls and from different severity β-thalassemia/Hb E patients. For example day 7 conditioned media from the culture of erythroid cells from severe β-thalassemia/Hb E patients showed significantly greater up-regulation of hepcidin expression at 3 and 6 hours as compared to media from the culture of normal control or mild β-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 4), where hepcidin expression was still suppressed at the last time point examined (48 hr post-treatment).

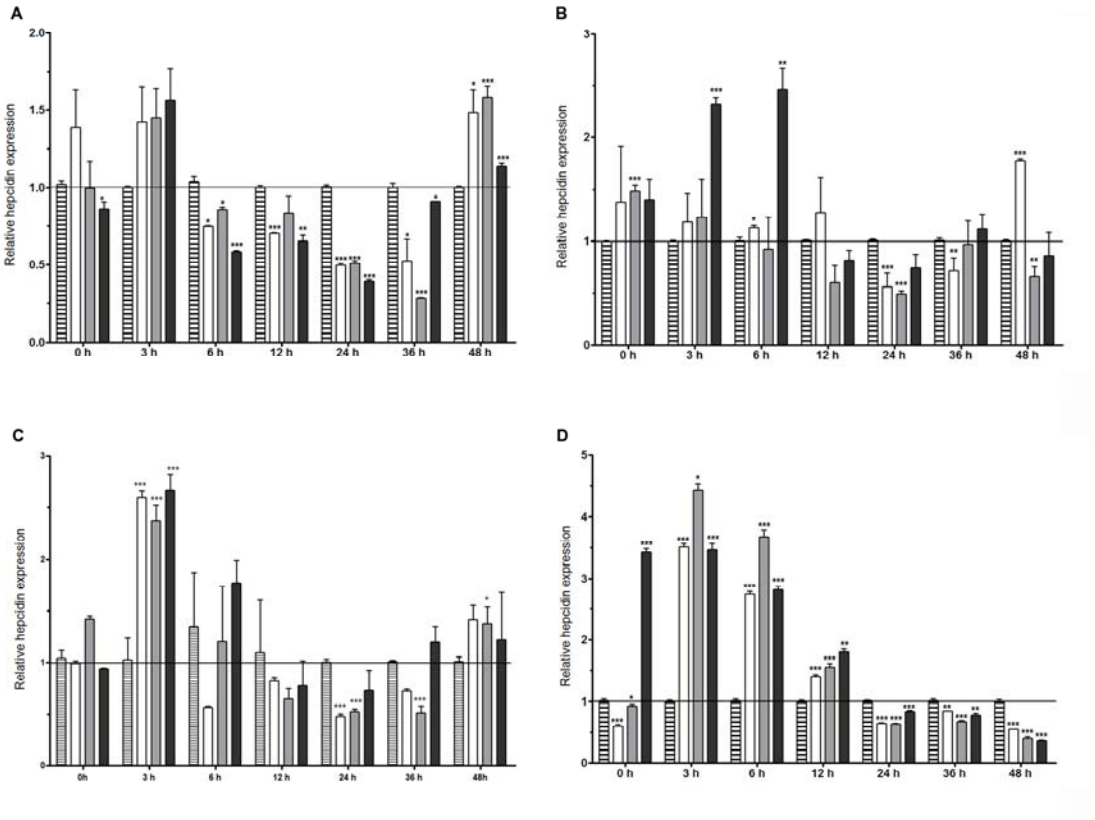

Figure 4. Hepcidin mRNA expression in HepG2 cells after treatment with erythroblast conditioned media. HepG2 cells were treated with day (A) 3, (B) 7, (C) 10 and (D) 14 conditioned media derived from culture of erythroid cells from normal controls (white bars), mild (grey bars) and severe (black bars) β°-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 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Molecular weight characterization

To undertake a preliminary characterization of the factor or factors mediating the suppression of hepcidin expression in liver cells, day 7, 10 and 14 conditioned media from the culture of erythroid cells from normal controls was size separated into three fractions (>100kDa, 50-100kDa, <50kDa) using centrifugal concentrators. The fractions were normalized back to original volume equivalents and used to treat HepG2 cells with quantitative PCR assay of hepcidin expression undertaken at 24 hr post-treatment. Results showed suppression of hepcidin expression in cells treated with the <50kDa fraction. Consistent with the previous results, media from all three days showed suppression of hepcidin expression. Interestingly, there was up-regulation of hepcidin expression by day 14 conditioned media in both the >100 kDa and 50-100kDa fractions.

To further investigate the factor suppressing hepcidin expression, day 14 conditioned media from the culture of erythroid cells from normal controls was separated into >100kDa, 50-100kDa, 30-50kDa, 3-10kDa and <3kDa fractions and after normalizing the volumes the fraction were used to treat HepG2 cells as previously. Cells treated with complete erythroid culture media (complete IMDM) were run in parallel as a control. At 24 hr post treatment the expression of hepcidin was determined as previously. Results (Figure 5) again showed increased expression in the >100kDa fraction and suppression of hepcidin was seen to occur in HepG2 cells treated with the <3kDa fraction. To confirm this result, day 14 media from the culture of erythroid cells from normal controls, mild and severe β-thalassemia patients was fractionated into >3kDa and <3kDa fractions and used to treat HepG2 cells in parallel with un-fractionated media and complete IMDM media treated HepG2 cells. Results (Figure 5) showed significant suppression of hepcidin expression in the <3kDa fraction. Suppression was also observed in the un-fractionated media as well as in the >3kDa fraction, but no suppression was seen in the complete IMDM treated cells.

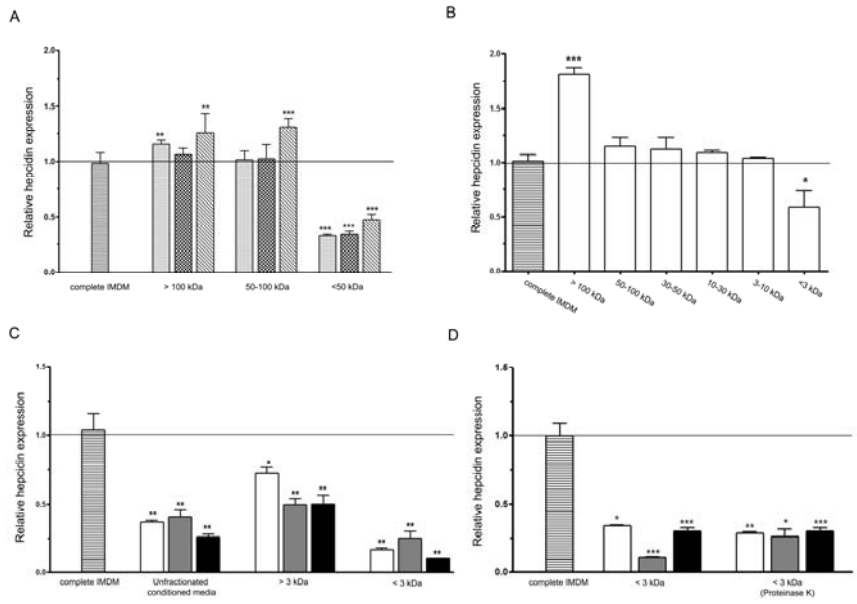

Figure 5. Hepcidin mRNA expression in HepG2 cells after treatment with size fractionated erythroblast conditioned media. A. HepG2 cells were treated with size fractionated day 7 (dotted bars), day 10 (chequered bars) or day 14 (diagonal lines) conditioned media derived from culture of erythroid cells from normal controls, or with complete IMDM (horizontal stripe bars). B. HepG2 cells were treated with size fractionated (as indicated) day 14 conditioned media derived from culture of erythroid cells from normal controls, or with complete IMDM (horizontal stripe bars). C. HepG2 cells were treated with size fractionated (as indicated) or complete day 14 conditioned media derived from culture of erythroid cells from normal controls (white bars), mild (grey bars) and severe (black bars) β°-thalassemia/ Hb E patients or with complete IMDM (horizontal stripe bars). (D) HepG2 cells were

treated with proteinase K treated or non-treated size fractionated (<3kDa) day 14 conditioned media derived from culture of erythroid cells from normal controls (white bars), mild (grey bars) and severe (black bars) β°-thalassemia/ Hb E patients or with complete IMDM (horizontal stripe bars). Each experiment was undertaken as three independent biological replicates with triplicate qPCR. Error bars represent SEM (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Preliminary characterization of the <3kda hepcidin suppression factor

The previous results have shown that cultured erythroid cells secrete a <3kDa factor that suppressed hepcidin expression in liver cells. Given that hepcidin is itself less than 3kDa (hepcidin is 2.7 kDa), we first determined whether hepcidin itself was able to suppress the expression of the hepcidin message, and HepG2 cells were therefore treated with hepcidin and the effect on expression determined at 24 hr post treatment. Results showed no suppression on hepcidin expression in treated cells. The <3kDa fraction from day 14 culture media from culture of erythroid cells from normal controls and mild and severe β-thalassemia/Hb E patients was digested with trypsin and the resultant peptides were analyzed by LC-MS/MS. Results (Table 1) showed peptides corresponding to larger proteins, with no common candidate between the fractions. Finally, to try to determine whether the factor was a protein, the <3kDa fraction was treated or not treated with proteinase K prior to addition to HepG2 cells and subsequent analysis of hepcidin expression. Results (Figure 5) showed no relief of suppression after proteinase K treatment.

Table 1. List of proteins identified from a < 3 kDa fraction of day 14 conditioned media from culture of erythroblasts from normal controls and mild and severe b^0-thalassemia/Hb E patients

Accession No.	Mass (Da)	Score	Matching peptides	Description
Less than 3 kDa of day 14 normal control conditioned media				
Q9Y4I1	216979	21	2	Unconventional myosin-Va [Homo sapiens]
Q06547	42571	17	1	GA-binding protein subunit beta-1 [Homo sapiens]
G3V0H7	72284	16	2	Putative solute carrier organic anion transporter family member 1B7 [Homo sapiens]
Q9UPN3	843033	15	4	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5 [Homo sapiens]
Less than 3 kDa of day 14 mild β^0-thalassemia/Hb E conditioned media				
Q86X95	52566	22	1	Corepressor interacting with RBPJ 1 [Homo sapiens]
P60903	11310	13	3	Protein S100-A10 [Homo sapiens]
Q8IZF0	202196	13	2	Sodium leak channel non-selective protein [Homo sapiens]
Less than 3 kDa of day 14 severe β^0-thalassemia/Hb E conditioned media				
O60312	169806	21	1	Probable phospholipid-transporting ATPase [Homo sapiens]

The thalassemia trait: protection against dengue virus

DENV-2 infection of erythroid precursor cells

To investigate the susceptibility of erythroid committed precursor cells, CD34+ HSCs were selected and cultured for 7 and 10 days under conditions that drive erythropoiesis (Leecharoenkiat et al., 2011; Lithanatudom et al., 2010; Wannatung et al., 2009) and infected with DENV at m.o.i. 50. At 48 h post infection (p.i.), the degree of cell infection was determined by flow cytometry. Results showed that nearly 80% of the cells infected on day 7 were infected, while approximately 40% of cells cultured for 10 days prior to infection were infected which is similar to the levels seen when day 4 cells (erythroid progenitor) were infected. We additionally analyzed infection of erythroid committed precursor cells from thalassemia carriers to DENV-2 infection and CD34+ HSCs were isolated from 3 individuals each of Hb E trait, β -thalassemia trait and α-thalassemia 1 trait and cultured for 7 or 10 days and infected with DENV-2, and samples were analyzed by flow cytometry at 48 h p.i. The results showed that erythroid committed precursor cells from all three traits were susceptible to infection, and, as with control erythroblast, higher levels of infection were seen in cells infected at day 7 as compared to day 10. While no difference was seen in the level of infection between erythroblasts infected on day 10 of culture, all three traits showed significantly lower levels of infection ($p<0.05$) as compared to normal control cells when day 7 cells were infected, with the lowest level of infection being seen in cells isolated from thalassemia carriers of α-thalassemia 1. We additionally determined whether normal control day 7 erythroid committed precursor cells were susceptible to infection with CHIKV using m.o.i. 0.1, 1 and 5 and the results revealed that these cells were not susceptible to CHIKV infection.

Differentiation of erythroid committed precursor cells

Culture of isolated CD34+ HSCs for 7 to 10 days results in a pool of cells at different stages of differentiation (proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts) as shown previously (Leecharoenkiat et al., 2011). To determine whether the reduced infection could result from altered differentiation of erythroid committed precursor cells from thalassemia carriers, the differentiation of normal control cells was compared to cells isolated from α-thalassemia 1 carriers. Day 7 and day 10 erythroid precursor cells from three β-thalassemia 1 carriers and three normal controls were stained with antibodies directed against CD71 and glycophorin A and analyzed by flow cytometry. Cell populations were gated to R1-R4 areas according to erythroid cell maturation as described by others (McGrath et al., 2008), and representative flow cytometry density plots are shown. No difference in differentiation was observed between day 7 erythroblasts from normal controls and α-thalassemia 1 carriers, with the majority of cells being proerythroblast (R1) and basophilic erythroblasts (R2). A significantly higher level of basophilic erythroblasts were seen in day 10 α-thalassemia 1 trait cells as compared to normal controls ($p= 0.013$), but as no difference in infection was seen in day 10 erythroblasts, the difference is unlikely to be of significance in the infection process.

DENV-2 replication in erythroid committed precursor cells

Evidence from flow cytometry is consistent with infection of erythroid committed precursor cells for both normal controls and thalassemia traits. However, the antibody used to determine the degree of infection is directed against a structural protein (the E protein) and therefore it is possible that positive cells reflect internalized virus without viral replication occurring. To exclude this possibility, cells were investigated for expression of dengue non-structural protein 1 (NS1) whose expression results from translation of the viral genomic RNA and is absolutely required for viral replication (Muller and Young, 2013). Day 7 and day 10 erythroblasts from normal controls and thalassemia carriers of Hb E, β-thalassemia and α-thalassemia 1 were either infected with DENV-2 or mock infected and at day 2 p.i. proteins were extracted and used in western analysis. Results (Figure 6) showed the clear expression of NS1 in all DENV-2 infected cells. DENV NS1 protein is found intracellularly and as a membrane associated and secreted hexameric form which is composed of heat labile amphipathic dimeric subunits (Gutsche et al., 2011; Pryor and Wright, 1993). To detect secreted DENV NS1, supernatants from day 7 mock and infected erythroblasts were separated by electrophoresis and subjected to western blot analysis after transfer to solid matrix support. Dimeric NS1 was observed in infected supernatants which were boiled at 80 ˚C and was not present in 100 ˚C boiled protein (Figure 6). Erythroblasts are cultured in medium containing human AB serum, and thus it was not possible to observe the monomeric form due to cross reaction from the secondary antibody used in the western blot. However, the blot was stripped and re-probed with secondary antibody only to serve as a loading control (Ig in Figure 6). Moreover, expression of NS1 protein was clearly evident in DENV-2 infected cells when normal infected and mock infected erythroblasts were stained with an anti-NS1 monoclonal antibody and an appropriate fluorescent secondary antibody and and examined under a confocal microscope (Figure 6). A greater number of NS1 positive cells were observed in day 7 infected erythroblasts than day 10 erythroblasts (Figure 6), consistent with the data on infection levels in these cells obtained by flow cytometry.

DENV-2 production from erythroid committed precursor cells

To show that DENV-2 infection of erythroid committed precursor cells was productive, day 7 and 10 erythroid committed precursor cells from three normal controls were infected with DENV-2 under standard conditions and virus production determined by standard plaque assay at multiple time points post infection. Virus titer of erythroblasts infected on day 7 showed a peak of virus production between 2 and 3 days post infection (Figure 6), after which virus titer gradually decreased. A similar trend was found when infecting day 10 erythroblasts, but the titer was lower than that observed for day 7 erythroblasts (Figure 6). In all cases a drop in virus titer as compared to the infecting titer was seen at 12 hours post infection, reflecting internalization of the virus into cells. Again, in all cases virus titer was higher at 24 h.p.i as compared to 12 h.p.i, reflecting *de novo* virus production.

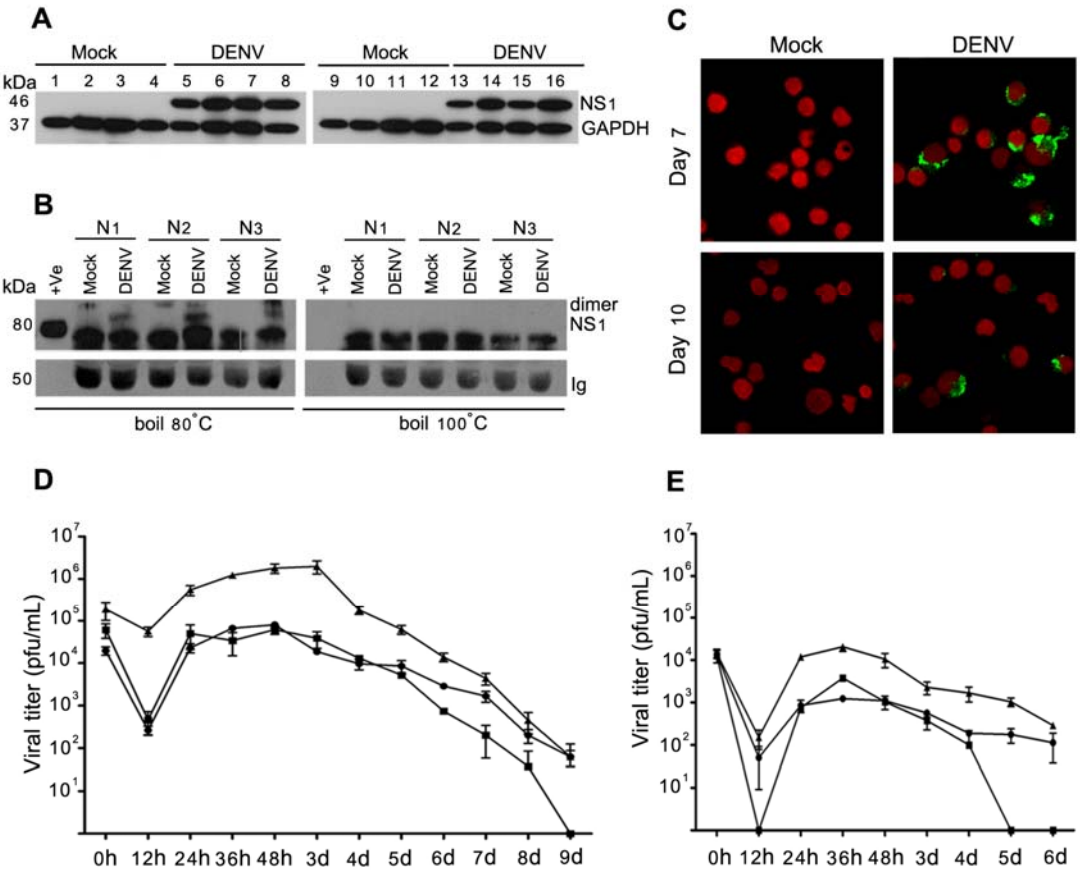

Figure 6. DENV-2 replication and production in erythroid precursor cells

(A) Erythroid committed precursor cells from normal control (lanes 1, 5, 9, 13), HbE trait (lanes 2, 6, 10, 14), β-thalassemia trait (lanes 3, 7, 11, 15) and α-thalassemia-1 trait (lanes 4, 8, 12, 16) were either infected (DENV) or mock infected (Mock) on day 7 and day 10 and analyzed after 48 h by western blotting for expression of DENV NS1 and GAPDH in a duplex analysis. (B) Supernatants were collected from day 7 erythroid precursor cells of three normal controls (N1, N2 and N3) at 48 after mock or DENV-2 infection. Protein was heated at either 80 ˚C or 100 ˚C and analyzed by western blot for detection secreted DENV NS1. DENV-2 infected erythroid cells were used as a positive control and heavy chain immunoglobulin (Ig) was used as an internal control. (C) Day 7 and day 10 normal erythroid committed precursor cells were either infected (DENV) or mock infected (Mock) and at 48 h.p.i. were stained with a mouse monoclonal anti-DENV NS1 protein antibody followed by a FITC-conjugated secondary antibody (green) and with To-Pro-3 iodide (red) as a nuclear counterstain. Cells were observed under a confocal microscope. Representative merged images are shown. (D and E) DENV production as assessed by standard plaque assay from day 7 (D) and day 10 (E) erythroid committed precursor cells after infection with DENV-2. Virus titer in the supernatant was detrmined at the times shown. Normal control 1 (solid circle), normal control 2 (solid square) and normal control 3 (solid triangle). Error bars represent SD in both panels.

Effects of DENV-2 infection on erythroid committed precursor cells

To determine the effects of DENV infection on erythroid committed precursor cells, day 7 normal control cells and α-thalassemia 1 trait cells were infected with DENV-2 and at 48 h p.i. examined for differentiation, cell number and cell apoptosis. While no difference in differentiation was seen in normal control erythroblasts, α-thalassemia 1 trait cells showed a significant difference in differentiation in response to infection with trait cells showing a significant reduction in state of differentiation. With respect to cell number, both normal control and α-thalassemia 1 trait day 7 infected cells showed a significant deficit in response to DENV infection as compared to uninfected cells, while only α-thalassemia 1 trait cells showed a deficit on cells infected on day 10. Cell apoptosis as assessed by propidium iodide and annexin V staining was increased in both day 7 infected normal control and α-thalassemia 1 trait erythroblasts as compared to mock infected, consistent with the deficit seen in cell number, while no significant difference was observed between day 10 infected and uninfected erythroblasts irrespective of trait status.

Erythroid differentiation stage and DENV-2 infection

To determine the susceptibility for DENV-2 infection of each erythroid cell stage, day 7 and day 10 erythroid precursor cells were generated from three normal controls, three β-thalassemia trait and 3 HbE trait carriers and were stained with an anti-DENV NS1 antibody followed by a biotinylated secondary antibody and the chromogenic signal finally developed using DAB. Cells were subsequently counterstained with Wright-Giemsa staining to allow differential counting of cells. A total of 500 cells from each sample were counted and scored for differentiation and the presence of NS1. The results showed that NS1 protein was found only in infected cells (Figure 7) and that the level of infection correlated with the earlier flow cytometry analysis in that infection rates were higher in day 7 cells as compared to day 10 cells and that day 7 erythroblasts from β-trait and HbE trait carriers were reduced as compared to normal control cells (Table 2). Proerythroblasts and basophilic erythroblasts were markedly more susceptible to DENV-2 infection, and susceptibility was reduced at the polychromatic erythroblast stage and dramatically dropped at orthochromatic erythroblasts (Table 2).

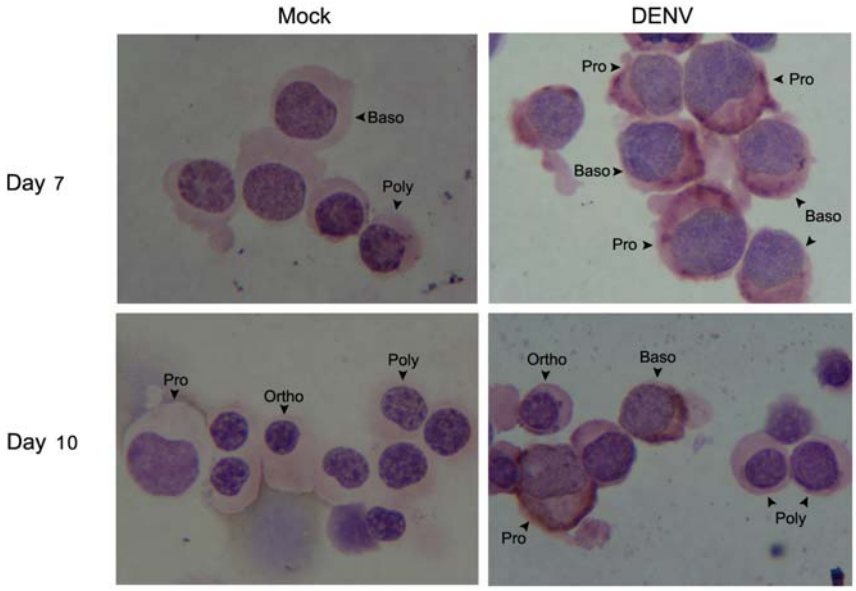

Figure 7. DENV infection and erythroid stage

Representative fields of DENV infected and mock infected day 7 and day 10 erythroblasts after immunochromogenic NS1 staining and counter staining with Wright-Giemsa stain. Cells were observed under a bright field microscope and DENV NS1 presents as a brown color in the cytoplasm. Identified cells are indicated as proerythroblast (Pro), basophilic erythroblast (Baso), polychromatic erythroblast (Poly) and orthochromatic erythroblast (Ortho).

Erythroid committed precursor cells and low passage DENV isolates

As several studies have shown that laboratory adapted DENVs can show behavior distinct from that of low passage DENV isolates (Chaichana et al., 2014; Diamond et al., 2000; Edgil et al., 2003), normal control day 7 erythroid committed precursor cells were mock infected or infected with DENV-2 high passage (16681) as used in the previous experiments and a low passage DENV-2 isolate and a low passage DENV-4 isolate and samples were analyzed by flow cytometry to determine percentage infection. Results showed high levels of infection with the laboratory adapted virus, but undetectable levels of infection with the two low passage isolates (Figure 8). To determine more accurately whether any cells had been infected by the low passage isolates, the normal control cells were stained with an anti-DENV NS1 antibody and counterstained with Wright-Giemsa staining as described previously. While infected cells were clearly evident when the infecting virus was the high passage, laboratory adapted strain, no cells showing a similar staining pattern were detected with the low passage strains. However rare scattered cells were positive for NS1 protein, but the morphology suggested that these cells were either dead or dying cells. In this analysis cells were counted before being cytospun onto the glass slides prior to staining and an apparent deficit was noted in cell number with cells exposed to the low passage isolates.

To determine if there was a high degree of apoptosis ongoing in the low passage isolate infections, day 7 erythroid committed precursor cells from both normal controls and hemoglobin E trait were therefore either mock infected or infected with the high and low passage isolates as in the earlier experiment. Cell death was assessed by propidium iodide and Annexin V staining and subsequent flow cytometry at 48 h.p.i. Results (Figure 8) showed significantly higher levels of cell death of normal control erythroid committed precursor cells infected with low passage DENV-2 as compared to laboratory adapted DENV-2 (Figure 8), and markedly, but not significantly higher levels of apoptosis in cells infected with low passage DENV-4 as compared to laboratory adapted DENV-2. Interestingly, both low passage isolates induced significantly higher levels of apoptosis in erythroid committed precursor cells from hemoglobin E trait as compared to the laboratory adapted virus (Figure 8). The level of apoptosis induced by the laboratory adapted DENV-2 was significantly higher in hemoglobin E trait cells than in normal control cells ($p=0.02$) consistent with the earlier observation with α-thalassemia 1 trait as compared to normal control.

The results from infection of erythroid committed precursor cells with low passage isolates of two different serotypes of rare scattered cells of being positive for NS1, but high levels of cell death are consistent with the induction of bystander cell death. Bystander cell death can be mediated by the induction of cytokines such a tumor necrosis factor alpha (TNF-α) capable of triggering apoptosis in surrounding, uninfected cells through the activation of death receptor mediated apoptosis and activation of caspase 8 (Gnesutta and Minden, 2003). To determine if this was the mechanism behind the high levels of cell death seen in response to infection with low passage isolates, erythroid committed precursor cells from normal controls were infected with low passage DENV-4 in the presence or absence of either an inhibitor of caspase 8 or an antibody directed against tumour necrosis factor receptor 1 (TNF R1), and at 48 hours post infection both the level of apoptosis and the cell number were determined. Pre-treatment of cells with a caspase 8 inhibitor both significantly reduced the level of apoptosis (Figure 8) and significantly increased the cell number (Figure 8) as compared to non-treated cells. Similarly, post treatment of infected cells with an antibody directed against TNF R1 significantly reduced the level of apoptosis (Figure 8) and significantly increased the cell number (Figure 8) as compared to untreated cells.

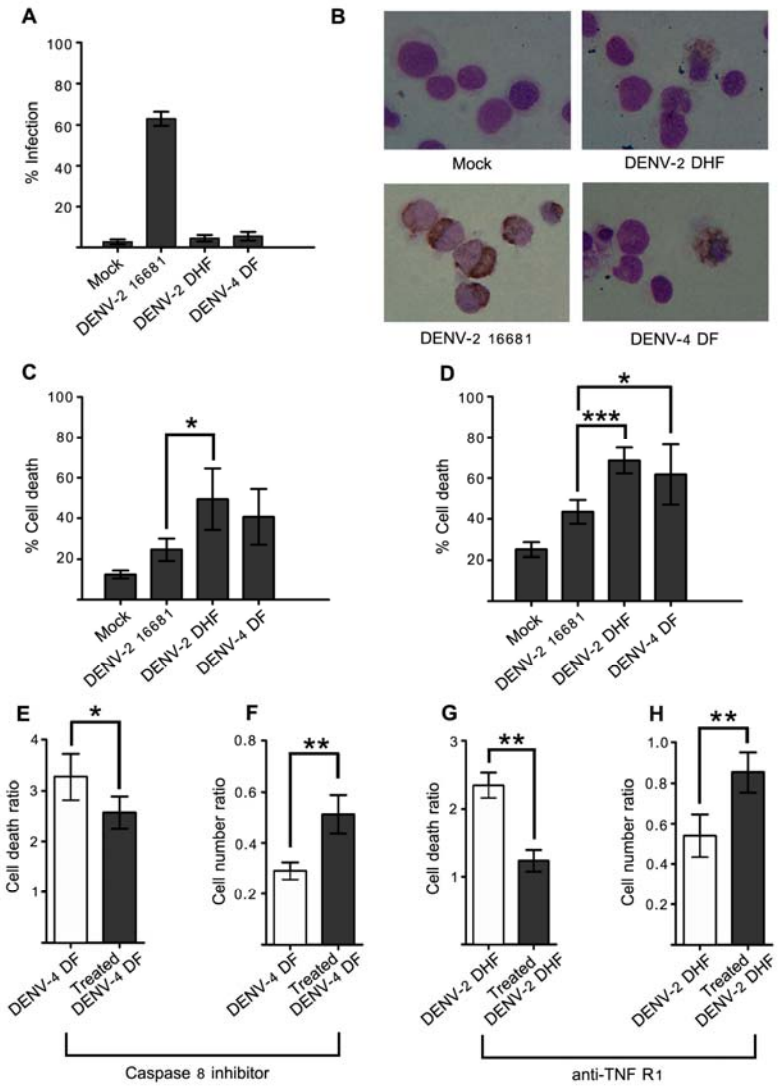

Figure 8. Clinical isolate DENV infection of erythroid committed precursor cells

The infection of DENV-2 DHF, DENV-4 DF, DENV-2 strain 16681 (positive control), and mock infected cells (negative control) of day 7 erythroid precursor cells from 3 normal controls (A) was assessed by flow cytometry. Both uninfected (Mock) and DENV infected erythroid precursor cells were stained with anti-DENV NS1 and counterstained with Wright-Giemsa staining (B). Cell death of day 7 erythroid precursor cells from 2 normal controls (C) and 3 HbE traits (D) at 48 h p.i. were evaluated for annexin V and propidium iodide staining by flow cytometry. Cell death (E,G) and cell number (F,H) of DENV-4 DF infection after 10 μM Caspase 8 inhibitor treatment (E,F) and DENV-2 DHF infection after 0.5 μg/mL anti-TNF-R1 treatment (G,H) were determined, all data were normalized against mock and treated mock. Error bars represent SD (*; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$).

The thalassemia trait: dysregulation of ribosome biosynthesis

The methylation status of three sites (1858, 4197 and 4506) in 28S rRNA extracted from whole blood was investigated in 18 normal controls and 15 β-thalassemia trait carriers by RT-qPCR. These sites were chosen for their localization within a key functional domain of the 28S rRNAs, *i.e*., the peptidyl transferase center [Demeshkina et al., 2012]. From quantitative PCR, melting curve analysis was used to confirmed the specificity of the amplification products (See Supplemental Figures). In addition, amplification cycle analysis verified that differentially amplified products were obtained from high and low dNTPs condition. The difference in amplification was calculated and the results are expressed as the methylation ratio. The results showed that two of the sites, at positions 1858 and 4506 were significantly hypermethylated in trait carriers (3.45±1.50 and 61.69±45.39 respectively) as compared to normal control samples (2.33±0.38 and 37.16±30.95 respectively; p = 0.0001 and 0.0075 respectively) while the third site at position 4197 showed no significant difference in methylation ratio between normal controls and trait carriers (3.73±1.01 and 4.16±1.66, respectively; p=0.39).

To provide direct evidence of ribosome biogenesis dysfunction in the pathologically important erythroid cells, the 2'-O-methylation status of rRNA from cultured erythroid cells was determined. To provide more information on the involvement of ribosome biogenesis in other haemoglobinopathies, in addition to cells from β-thalassemia trait carriers, cells from α-thalassemia 1 and haemoglobin E (HbE) trait carriers were also examined. CD34+ HSCs were therefore isolated and cultured to a time point after globin gene expression onset, namely day 10 of culture [Leecharoenkiat et al., 2011c; Wojda et al., 2002]. The methylation results showed that site 4506 was significantly hypermethylated in heterozygous β-thalassaemia (81.86±16.52) but not in α-thalassaemia 1 or in heterozygous Hb E when compared to normal controls (46.29±18.39, p = 0.019). The other 2 methylation sites examined at positions 4197 and 1858 showed no significant difference between any groups (Figure 2). At position 1858 is significantly hypermethylated in heterozygous β-thalassaemia of RNA derived from peripheral blood when compared to normal. However, this position was relatively increased methylation level in heterozygous β-thalassaemia (3.48±0.48) of RNA sample derived from day 10 erythroid cells when compared to normal (3.05±0.44). This result confirms the dysregulation of ribosome biogenesis in people with thalassemia trait.

Conclusions

While ineffective erythropoiesis is the underlying pathobiology of β-thalassemia disease, the resultant iron overloading is the main physiological deficit. Iron export from cells is controlled by the expression of the ferroportin protein and surprisingly developing erythroid precursor cells, which require iron for hemoglobinization, also express the ferroportin iron export protein. This study showed that there is significant dysregulation of ferroportin expression in differentiating erythroid precursor cells from severe β^0-thalassemia/Hb E patients, and that this leads to the inability of these cells to maintain iron homeostasis, possibly contributing to ineffective erythropoiesis. Ferroportin is post-translationally regulated by the peptide hormone hepcidin, and dysregulation of the hepcidin-ferroportin axis has implications for other diseases besides thalassemia. This study developed and validated a system to investigate the mechanism by which erythroid cells signal to liver cells to suppress hepcidin expression, allowing iron to be released for hemoglobinization. Interestingly the results point to a novel, sub-3kD mediator of hepcidin expression.

Thalassemia is highly prevalent in Thailand, with up to 50% of the population being trait carriers in some parts of the country. While a heterozygous trait advantage for thalassemia has been shown for malaria, any benefit for other diseases has not been shown. Dengue virus (DENV) is endemic in Thailand and several bone marrow derived cells are susceptible to DENV infection. This study showed that the normally bone marrow resident erythroid precursor cells were susceptible to DENV infection, but that cells from trait carriers were less susceptible. Markedly however cells from trait carriers showed significantly increased bystander apoptosis mediated by TNF-alpha, suggesting that thalassemia trait status may provide some advantage in DENV infection. How erythroid precursor cells from thalassemia trait carriers are less susceptible to DENV remains unclear, but we additionally demonstrated that ribosome biogenesis in trait carrier erythroid precursor cells is dysregulated as compared to normal erythroid precursor cells which may provide a mechanistic answer. Much of the work undertaken in this project is highly novel, and it is hoped that these new insights will lead to improved treatment options for thalassemia.

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Output: Papers

1. Sornjai W, Khungwanmaythawee K, Svasti S, Fucharoen S, Wintachai P, Yoksan S, Ubol S, Wikan N, Smith DR*. Dengue virus infection of erythroid precursor cells is modulated by both thalassemia trait status and virus adaptation. Virology 2014; 471-473:61-71. Impact factor = 3.321 (**ที่มา :** Journal Citation Reports, 2014).

2. Sornjai W, Jaratsittisin J, Khungwanmaythawee K, Svasti S, Fucharoen S, Lithanatudom P, Smith DR*. Dysregulation of ferroportin gene expression in β0-thalassemia/Hb E disease. Ann Hematol. Ann Hematol 2016;95: 387-396. Impact factor = 2.63 (**ที่มา :** Journal Citation Reports, 2014).

3. Khungwanmaythawee K, Sornjai W, Paemanee A, Jaratsittisin J, Fucharoen S, Svasti S, Lithanatudom P, Roytrakul S, Smith DR*. Mitochondrial changes in b0-thalassemia/Hb E disease. PLoS One 2016; 11: e0153831. Impact factor = 3.057 (**ที่มา :** Journal Citation Reports, 2015).

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6. Sornjai W, Lithanatudom P, Erales J, Joly P, Francina, Hacot S, Fucharoen S, Svasti S, Diaz JJ, Mertani HC, Smith DR*. Hypermethylation of 28S ribosomal RNA in b-thalassemia trait carriers. Int J Biol Macromol 2017; 94:728-734. Impact factor = 3.138 (**ที่มา :** Journal Citation Reports, 2015).

OUTPUT: ABSTRACTS PRESENTED AT SCIENTIFIC MEETINGS

Invited lecture

1. Duncan R. Smith, Amporn Leecharoenkiat, Kornpat Khungwanmaythawee, Wannapa Sornjai, Suthat Fucharoen, Saovaros Svasti, Atchara Paemanee and Sittiruk Roytrakul. Application of global and organelle enriched proteomic analysis to understanding β-thalassemia/HbE disease. 7th AOHUPO Congress and 9th International Symposium of the Protein Society of Thailand. Miracle Grand Convention Hotel, Bangkok, Thailand. (6-8 August 2014).

Oral Presentations

1. Janejira Jaratsittisin, Wannapa Sornjai, Kornpat Khungwanmaythawee, Saovaros Svasti, Suthat Fucharoen, Sittiruk Roytrakul and Duncan r. Smith. Regulation of hepcidin expresstion by erythroblast conditioned media. ASEAN Congress on Medical Biotechnology and Molecular Biosciences 2015, 8-9 October 2015, Arnoma Grand Hotel, Bangkok, Thailand.

2. Duncan R. Smith, Wannapa Sornjai, Janejira Jaratsittisin, Kornpat Khungwanmaythawee, Saovaros Svasti, Suthat Fucharoen and Sittiruk Roytrakul. Analysis of aspects of the hepcidin-ferroportin axis in β-thalassemia/Hb E disease. TRF-OHEC Annual Congress 2016. (การประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. ครั้งที่ 15). 6th-8th January 2016. The Regent Cha Am Beach Resort, Thailand.

3. Duncan R. Smith, Wannapa Sornjai, Janejira Jaratsittisin, Kornpat Khungwanmaythawee, Saovaros Svasti, Suthat Fucharoen and Sittiruk Roytrakul. Analysis of aspects of the hepcidin-ferroportin axis in β-thalassemia/Hb E disease. การประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. ครั้งที่ 16. 11th-13th January 2017. The Regent Cha Am Beach Resort, Thailand.

Poster presentations

1. Kornpat Khungwanmaythawee, Wannapa Sornjai, Atchara Paemanee, Chartchai Chaichana, Kamonlak Leecharoenkiat, Sittiruk Roytrakul, Suthat Fucharoen, Saovaros Svasti and Duncan R. Smith. The Role of Mitochondria in Beta-thalassemia/Hb E Disease. "19th Biological Sciences Graduate Congress" National University of Singapore, Singapore. (12-13 December 2014)

2. Wannapa Sornjai, Janejira Jaratsittisin, Kornpat Khungwanmaythawee, Saovaros Svasti, Suthat Fucharoen and Duncan R. Smith. The expression of ferroportin in beta-thalassemia/Hb E erythroblasts. "International Congress on Chemical, Biological and Environmental Sciences (ICCBES)" 7-9th May, 2015. Kyoto, Japan.

3. Janejira Jaratsittisin, Wannapa Sornjai, Kornpat Khungwanmaythawee, Saovaros Svasti, Suthat Fucharoen, Sittiruk Roytrakul and Duncan R. Smith. Regulation of hepcidin expression by

erythroblast conditioned media. “International Congress on Chemical, Biological and Environmental Sciences (ICCBES)” 7-9th May, 2015. Kyoto, Japan.

4. Wannapa Sornjai, Janejira Jaratsittisin, Kornpat Khungwanmaythawee, Saovaros Svasti, Suthat Fucharoen and Duncan R. Smith. Basal expression of erythroid regulators mRNA of β-thalassemia/Hb E erythroblasts. the 36th World Congress of the International Society of Hematology. 18-21 April, 2016. Glasgow, United Kingdom.

Students.

The following students completed their studies during the course of this grant:

Doctor of Philosophy (Ph.D.)

Ms. Kornpat Khungwanmaythawee Ph.D. (Program in Molecular Genetics and Genetic Engineering) Institute of Molecular Biosciences, Mahidol University. (Completed June 2016)

Ms. Wannapa Sornjai Ph.D. (Program in Molecular Genetics and Genetic Engineering). Institute of Molecular Biosciences, Mahidol University. (Completed February 2017)

Master of Science (M.Sc.)

Ms. Janejira Jaratsittisin M.Sc. (Program in Molecular Genetics and Genetic Engineering). Institute of Molecular Biosciences, Mahidol University. (Completed June 2016)

Appendix

Virology 471-473 (2014) 61–71

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Dengue virus infection of erythroid precursor cells is modulated by both thalassemia trait status and virus adaptation

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ABSTRACT

Dengue is the most significant arthropod borne viral disease worldwide, and infection with the dengue virus causes a wide range of symptoms in humans, including bone marrow suppression. While the target cells of the virus remain poorly characterized, cells of the myeloid lineage have been shown to be important mediators of the disease. This study sought to determine whether erythroid precursor cells were susceptible to dengue virus infection, and whether erythroid cells from thalassemia trait carriers showed any protection against infection. Infection with a laboratory adapted high passage DENV-2 resulted in high levels of infection during certain stages of differentiation, and cells derived from thalassemia trait carriers showed significantly reduced susceptibility to dengue virus infection. Infection with low passage isolates resulted in only scattered cells showing evidence of infection, but high bystander apoptosis that was reduced by both a caspase 8 inhibitor and anti-tumor necrosis factor 1 receptor antibodies.

Introduction

Dengue virus (DENV) is recognized as the most common arthropod borne human viral pathogen and is endemic in many tropical and subtropical countries. The virus belongs to family *Flaviviridae*, genus *Flavivirus* and possesses a positive sense single stranded RNA genome. Four serotypes of DENV have been classified, namely DENV-1, DENV-2, DENV-3 and DENV-4. Human infection with the virus occurs after the bite of an infected female mosquito and results in a broad range of clinical manifestations including fever, rash and headache as well as muscle and joint pain (Gubler, 1998). Early bone marrow suppression, thrombocytopenia and leukopenia are clinical hallmarks in dengue patients, but the etiology remains unclear (Bierman and Nelson, 1965; Srichaikul and Nimmannitya, 2000). Infection of the bone marrow has been suggested as a contributing factor as this compartment is the major site for hematopoiesis and bone marrow infection was found in *in vivo* studies of non-human primates, and it was proposed that megakaryocytes are the major target for DENV in this compartment (Noisakran et al., 2012).

Pluripotent hematopoietic stem cells (HSCs) located in the bone marrow give rise to lymphoid (natural killer (NK), T and B cells) and myeloid (granulocyte, monocyte, dendritic, erythrocyte and megakaryocyte) cells (Gunsilius et al., 2001), and several cells of the myeloid lineage including dendritic cells (Wu et al., 2000), monocytes (Scott et al., 1980) and megakaryocytes (Noisakran et al., 2012) have been shown to be susceptible to dengue virus infection. Mature erythroid cells arise from hematopoietic stem cells which give rise to early erythroid committed progenitor cells (burst forming units-erythroid or BFU-E) which subsequently give rise to late erythroid progenitor cells (colony forming units-erythroid or CFU-E) which further differentiate to erythroid committed precursors which consist of a series of erythroblastic cells (proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts and reticulocytes) which finally develop into mature erythrocytes (Gunsilius et al., 2001). While previous *in vitro* studies have shown that hematopoietic progenitor cells are susceptible to DENV infection and inhibition of cell proliferation has been observed (Murgue et al., 1997; Nakao et al., 1989), no study has to date investigated the susceptibility to DENV infection of the more differentiated erythroid precursor cells.

Erythroid cells have been well characterized as a target for mosquito borne protozoan parasite *Plasmodium* spp., which are responsible for the disease malaria (Garcia, 2010) and the protective effects of hemoglobin variants or globin gene deletions has been well

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established (Taylor et al., 2012). For example heterozygous sickle cell traits have been well characterized as providing a protective advantage against malaria in endemic areas (Aidoo et al., 2002; Allison, 1954; Piel et al., 2010; Williams et al., 2005). Similarly, α^+-thalassemia traits have been proposed as another determinant that may provide protection against severe malaria (Enevold et al., 2007; Mockenhaupt et al., 2004; Wambua et al., 2006). These studies support the malaria hypothesis which proposes that it is the selective pressure provided by the malaria parasite that maintains the sickle cell and thalassemia traits in populations (Clegg and Weatherall, 1999). In some areas of the world, and particularly in Southeast Asia, thalassemia traits are found in the population at extremely high levels (Fucharoen and Winichagoon, 1992). In parts of Thailand the hemoglobin variant Hb E is found in up to 50% of the population and other traits show similarly high penetrance (Fucharoen and Winichagoon, 1992). It is possible therefore that other mechanisms besides malaria are providing selective pressure on maintaining these traits in the population. This study sought to determine whether erythroid precursor cells were susceptible to DENV infection, and whether thalassemia traits resulted in any modulation of DENV infectivity.

Materials and methods

Cells and virus

LLC-MK_2 cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithesburg, MD) supplemented with 5% heat inactivated fetal bovine serum (FBS; Gibco BRL) and 100 units/mL of penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria). The human erythroleukemia cell line K562 was grown in RPMI-1640 (Gibco BRL) supplemented with 10% FBS and 100 units/mL of penicillin/streptomycin. Both cell lines were incubated at 37 °C with 5% CO_2. Dengue virus serotype 2 (DENV-2; strain 16681; GenBank accession number M84727.1), Dengue virus serotype 2 (DENV-2 DHF; DENV-2/THAI/NS1-141/2006; GenBank Accession number KM519587) and Dengue virus serotype 4 (DENV-4 DF; DENV-4/THAI/NS1-058/2006; GenBank accession number KM519591) were used in this study. DENV-2/THAI/NS1-141/2006 and DENV-4/THAI/NS1-058/2006 were passaged 3 times through C6/36 cells prior to use in this study. All dengue viruses were propagated in C6/36 cells (ATCC CRL-1660) and chikungunya virus (CHIKV) ECSA 226V genotype was propagated in Vero (ATCC: CCL-81) cells as described elsewhere (Wikan et al., 2012). The supernatants were partially purified by centrifugation and stored at −80 °C as viral stock. All virus titers were determined by standard plaque assay on LLC-MK_2 cells or Vero cells as previously described (Sithisarn et al., 2003).

Sample collection and 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. Complete blood count (CBC) indices, hemoglobin typing and α-globin gene genotyping were used to classify thalassemia carriers and normal controls. Fifty milliliter of peripheral blood was taken from at least three individuals per group which were classified either as normal subject, β-thalassemia trait, Hb E trait or α-thalassemia 1 trait. CD34+ hematopoietic stem cells (HSCs) were isolated from peripheral blood samples and cultured under conditions that produced differentiation in the erythroid lineage as described in previously (Lithanatudom et al., 2010; Wannatung et al., 2009) and see more detail in Supplemental materials and methods. Under these growth conditions more than 95% of cells show erythroblast morphology as established previously (Wannatung et al., 2009). Cell numbers were established by trypan blue exclusion assay and counting using a cell counting chamber.

Virus infection

Cells (K562 cells or erythroid committed progenitor or precursor cells) were cultured in 6-well culture plates or 12-well culture plates under standard conditions and were infected with DENV-2 strain 16681 at various multiplicity of infection (m.o.i.) and DENV-2 DHF and DENV-4 DF at m.o.i. 50. Some normal erythroid committed precursor cells were infected with CHIKV at m.o.i. 0.1, 1 and 5. Cells and virus were incubated for 2 h in normal medium without serum albumin with occasional agitation after which complete medium was added and cells were incubated under standard conditions until required. In some experiments DENV-2 was pre-incubated for 1 h at 4 °C with a 1:100 dilution of a mouse monoclonal anti-DENV E protein antibody produced by hybridoma HB114 (Henchal et al., 1982) before addition to cells as described elsewhere (Klomporn et al., 2011). For viral production measurement, after 2 h of incubation, unbound virus was washed 4 times with IMDM medium without serum albumin. Supernatant was collected at several time points and virus titer was measured.

Flow cytometry, indirect immunofluorescence assay and immunocytochemical staining

Flow cytometry and indirect immunofluorescence were undertaken essentially as described elsewhere (Leecharoenkiat et al., 2011; Lithanatudom et al., 2010; Panyasrivanit et al., 2009). Immunocytochemical staining was performed using a Vectastain ABC kit following the manufacturer's protocol and counterstained with Wright–Giemsa staining. For full details, see file Supplemental materials and methods.

Western blot analysis

Supernatant was collected from DENV-2 infected cells. Total protein was extracted from mock infected and infected cells. Proteins were subjected to western blot analysis exactly as described previously (Leecharoenkiat et al., 2011). For further details, see file Supplemental materials and methods.

Caspase 8 inhibition

Day 7 erythroid precursor cells were pretreated with or without 10 μM of Caspase 8 inhibitor (Z-Ile-Glu(O-ME)-Thr-Asp(O-Me) fluoromethyl ketone; Sigma-Aldrich, St. Louis, MO) and incubated at 37 °C, 5% CO_2 with agitation for 2 h. Incubated cells were infected with DENV-4 DF with or without 10 μM of Caspase 8 inhibitor. After 2 h of viral incubation, complete IMDM medium with or without a final concentration of 10 μM of Caspase 8 inhibitor was added and cells were subsequently incubated at 37 °C with 5% CO_2 for 48 h.

TNF-R1 receptor blocking

Erythroid precursor cells were infected with DENV-2 DHF under the standard protocol and at 6 h post infection the medium was supplemented an anti-TNF-R1 (sc-7895; Santa Cruz Biotechnology Inc., Dallas, TX) antibody to final concentrations of 5 μg/mL. An identical amount of antibody was added at 24 h post infection. Treated and untreated cells were incubated under standard conditions.

Statistical analysis

Statistical analysis was performed by using PASW statistics version 18 (SPSS Inc., Chicago, IL). Data was analyzed by independent or paired sample *t*- tests as appropriate and a p value < 0.05 was considered as significant.

Results

DENV-2 infection of K562 cells and normal erythroid cells

The human erythromyeloblastoid leukemia cell line K562 is frequently used as a model for erythroid cell studies, and the cells can give rise to erythroid cells under appropriate induction (Osti et al., 1997; Rutherford et al., 1979). We therefore initially validated the susceptibility of this cell line to DENV infection as has been shown by others (Goncalvez et al., 2007) under both conditions of direct infection and under conditions of ADE infection (in which entry is mediated through the Fc receptor). Cells were therefore either directly exposed to DENV, or exposed to a DENV/antibody complex at m.o.i. of 1, 10 and 50 and after 48 h the percentage of infected cells was determined by flow cytometry. Results showed that K562 cells were able to be directly infected by DENV, and that significantly increased levels of infection were seen when the DENV was complexed with a non-neutralizing anti-DENV E protein antibody (Fig. 1A), consistent with the reports of others (Goncalvez et al., 2007).

We next determined the susceptibility of selected normal control CD34+ HSCs from two donors that had been cultured for 4 days (which corresponds to the erythroid committed progenitor stage) to be infected by DENV under conditions of direct infection and ADE infection with infectivity determined by flow cytometry as above at 48 h post infection. Results (Fig. 1B) showed infection of day 4 erythroid progenitor cells that increased with increasing m.o.i. between 1 and 50, and that no increase of infectivity was seen when cells were infected under conditions of ADE, suggesting that infection occurs by direct infection, and not through Fc receptor mediated internalization. These results are consistent with the earlier observations of others (Murgue et al., 1997; Nakao et al., 1989).

DENV-2 infection of erythroid precursor cells

To investigate the susceptibility of erythroid committed precursor cells, CD34+ HSCs were selected and cultured for 7 and 10 days under conditions that drive erythropoiesis (Leecharoenkiat et al., 2011; Lithanatudom et al., 2010; Wannatung et al., 2009) and infected with DENV at m.o.i. 50. At 48 h post infection (p.i.), the degree of cell infection was determined by flow cytometry. Results (Fig. 1C and D) showed that nearly 80% of the cells infected on day 7 were infected, while approximately 40% of cells cultured for 10 days prior to infection were infected which is similar to the levels seen when day 4 cells (erythroid progenitor) were infected. We additionally analyzed infection of erythroid committed precursor cells from thalassemia carriers to DENV-2 infection and CD34+ HSCs were isolated from 3 individuals each of Hb E trait, β-thalassemia trait and α-thalassemia 1 trait and cultured for 7 or 10 days and infected with DENV-2, and samples were analyzed by flow cytometry at 48 h p.i. The results showed that erythroid committed precursor cells from all three traits were susceptible to infection, and, as with control erythroblast, higher levels of infection were seen in cells infected at day 7 as compared to day 10 (Fig. 1C and D). While no difference was seen in the level of infection between erythroblasts infected on day 10 of culture (Fig. 1D), all three traits showed significantly lower levels of infection ($p < 0.05$) as compared to normal control cells when day 7 cells were infected (Fig. 1C), with the lowest level of infection being seen in cells isolated from thalassemia carriers of α-thalassemia 1. We additionally determined whether normal control day 7 erythroid committed precursor cells were susceptible to infection with CHIKV using m.o.i. 0.1, 1 and 5 and the results revealed that these cells were not susceptible to CHIKV infection (data not shown).

Differentiation of erythroid committed precursor cells

Culture of isolated CD34+ HSCs for 7 to 10 days results in a pool of cells at different stages of differentiation (proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts) as shown previously (Leecharoenkiat et al., 2011). To determine whether the reduced infection could result from altered differentiation of erythroid committed precursor cells from thalassemia carriers, the differentiation of normal control cells was compared to cells isolated from α-thalassemia 1 carriers. Day 7 and day 10 erythroid precursor cells from three α-thalassemia 1 carriers and three normal controls were stained with antibodies directed against CD71 and glycophorin A and analyzed by flow cytometry. Cell populations were gated to R1-R4 areas according to erythroid cell maturation as described by others (McGrath et al., 2008), and representative flow cytometry density plots are shown (Fig. 1E). No difference in differentiation was observed between day 7 erythroblasts from normal controls and α-thalassemia 1 carriers (Fig. 1F), with the majority of cells being proerythroblast (R1) and basophilic erythroblasts (R2). A significantly higher level of basophilic erythroblasts were seen in day 10 α-thalassemia 1 trait cells as compared to normal controls ($p = 0.013$; Fig. 1G), but as no difference in infection was seen in day 10 erythroblasts, the difference is unlikely to be of significance in the infection process.

DENV-2 replication in erythroid committed precursor cells

Evidence from flow cytometry is consistent with infection of erythroid committed precursor cells for both normal controls and thalassemia traits. However, the antibody used to determine the degree of infection is directed against a structural protein (the E protein) and therefore it is possible that positive cells reflect internalized virus without viral replication occurring. To exclude this possibility, cells were investigated for expression of dengue non-structural protein 1 (NS1) whose expression results from translation of the viral genomic RNA and is absolutely required for viral replication (Muller and Young, 2013). Day 7 and day 10 erythroblasts from normal controls and thalassemia carriers of Hb E, β-thalassemia and α-thalassemia 1 were either infected with DENV-2 or mock infected and at day 2 p.i. proteins were extracted and used in western analysis. Results (Fig. 2A) showed the clear expression of NS1 in all DENV-2 infected cells. DENV NS1 protein is found intracellularly and as a membrane associated and secreted hexameric form which is composed of heat labile amphipathic dimeric subunits (Gutsche et al., 2011; Pryor and Wright, 1993). To detect secreted DENV NS1, supernatants from day 7 mock and infected erythroblasts were separated by electrophoresis and subjected to western blot analysis after transfer to solid matrix support. Dimeric NS1 was observed in infected supernatants which were heated at 80 °C and was not present in 100 °C boiled protein (Fig. 2B). Erythroblasts are cultured in medium containing human AB serum, and thus it was not possible to observe the monomeric form due to cross reaction from the secondary antibody used in the western blot. However, the blot was stripped and re-probed with secondary antibody only to serve as a loading control (Ig in Fig. 2B). Moreover, expression of NS1 protein was clearly evident in DENV-2 infected cells when normal infected and mock infected erythroblasts were stained with an anti-NS1 monoclonal antibody and an

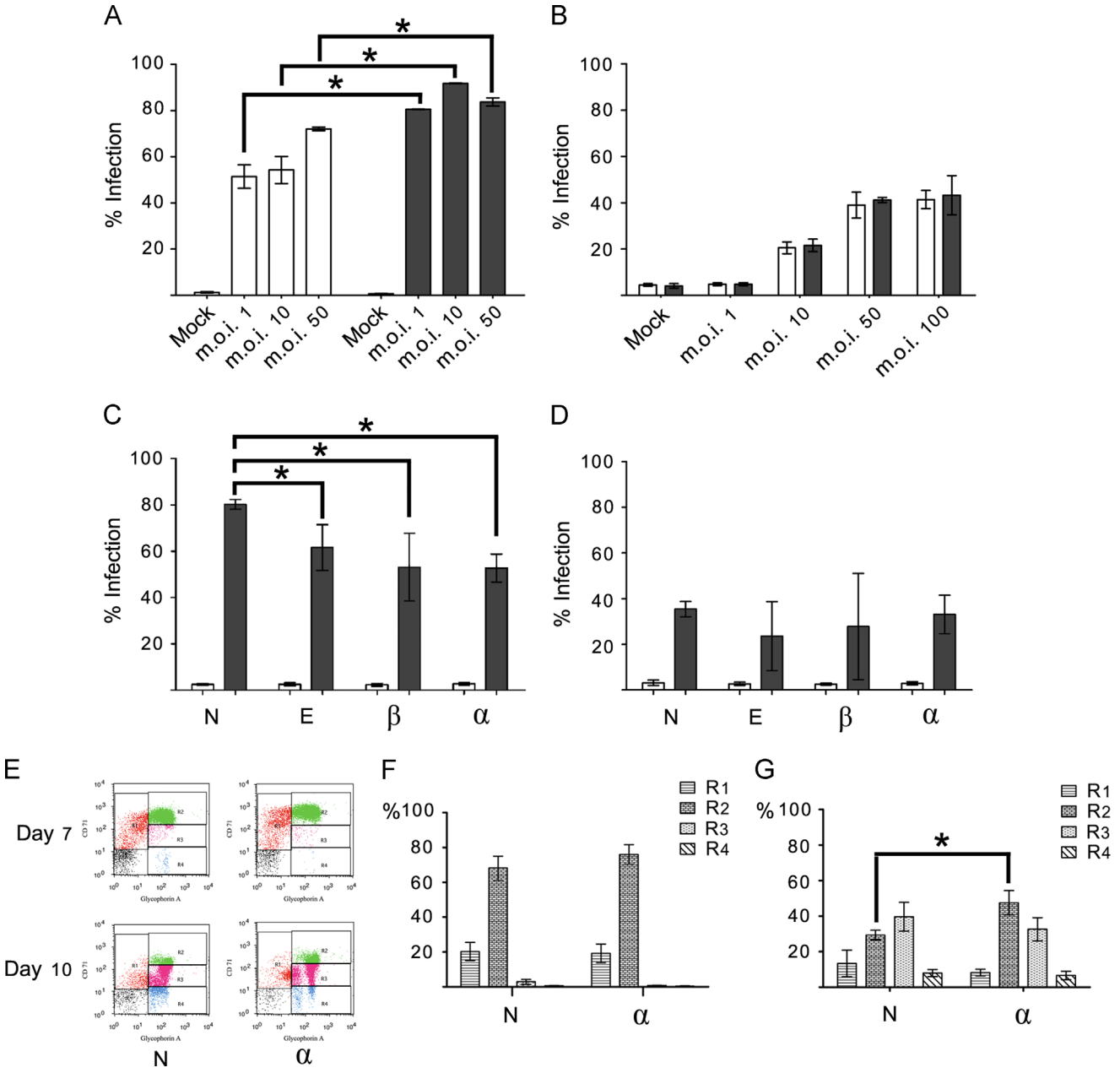

Fig. 1. DENV-2 infection and differentiation of erythroid cells. Direct (open bars) and ADE mediated (solid bars) DENV-2 infection of K562 cells (A) and day 4 erythroid progenitor cells (B) as assessed by flow cytometry. DENV-2 infection (solid bars) and mock infection (open bars) of day 7 (C) and day 10 (D) erythroid committed precursor cells from normal controls (N), Hb E trait (E), β-thalassemia trait (β) and α-thalassemia-1 trait (α). Differentiation of day 7 and day 10 normal control and α-thalassemia-1 trait erythroid committed precursor cells was assessed by flow cytometry after staining with antibodies directed against CD 71 and glycophorin A. Representative flow cytometry density plots are shown (E). Regions were gated as 1 (predominantly proerythroblasts), 2 (predominantly basophilic erythroblasts), 3 (predominantly polychromatophilic erythroblasts) and 4 (predominantly orthchromatophilic erythroblasts) as shown and data from three experiments was plotted as histograms for day 7 (F) and day 10 (G). Error bars represent SD (*; $p < 0.05$).

appropriate fluorescent secondary antibody and examined under a confocal microscope (Fig. 2C). A greater number of NS1 positive cells were observed in day 7 infected erythroblasts than day 10 erythroblasts (Fig. 2C), consistent with the data on infection levels in these cells obtained by flow cytometry.

DENV-2 production from erythroid committed precursor cells

To show that DENV-2 infection of erythroid committed precursor cells was productive, day 7 and 10 erythroid committed precursor cells from three normal controls were infected with DENV-2 under standard conditions and virus production determined by standard plaque assay at multiple time points post infection. Virus titer of erythroblasts infected on day 7 showed a peak of virus production between 2 and 3 days post infection (Fig. 2D), after which virus titer gradually decreased. A similar trend was found when infecting day 10 erythroblasts, but the titer was lower than that observed for day 7 erythroblasts (Fig. 2E). In all cases a drop in virus titer as compared to the infecting titer was seen at 12 h post infection, reflecting internalization of the virus into cells. Again, in all cases virus titer was higher at 24 h.p.i as compared to 12 h.p.i, reflecting *de novo* virus production.

Effects of DENV-2 infection on erythroid committed precursor cells

To determine the effects of DENV infection on erythroid committed precursor cells, day 7 normal control cells and α-thalassemia 1 trait cells were infected with DENV-2 and at 48 h p.i. examined for

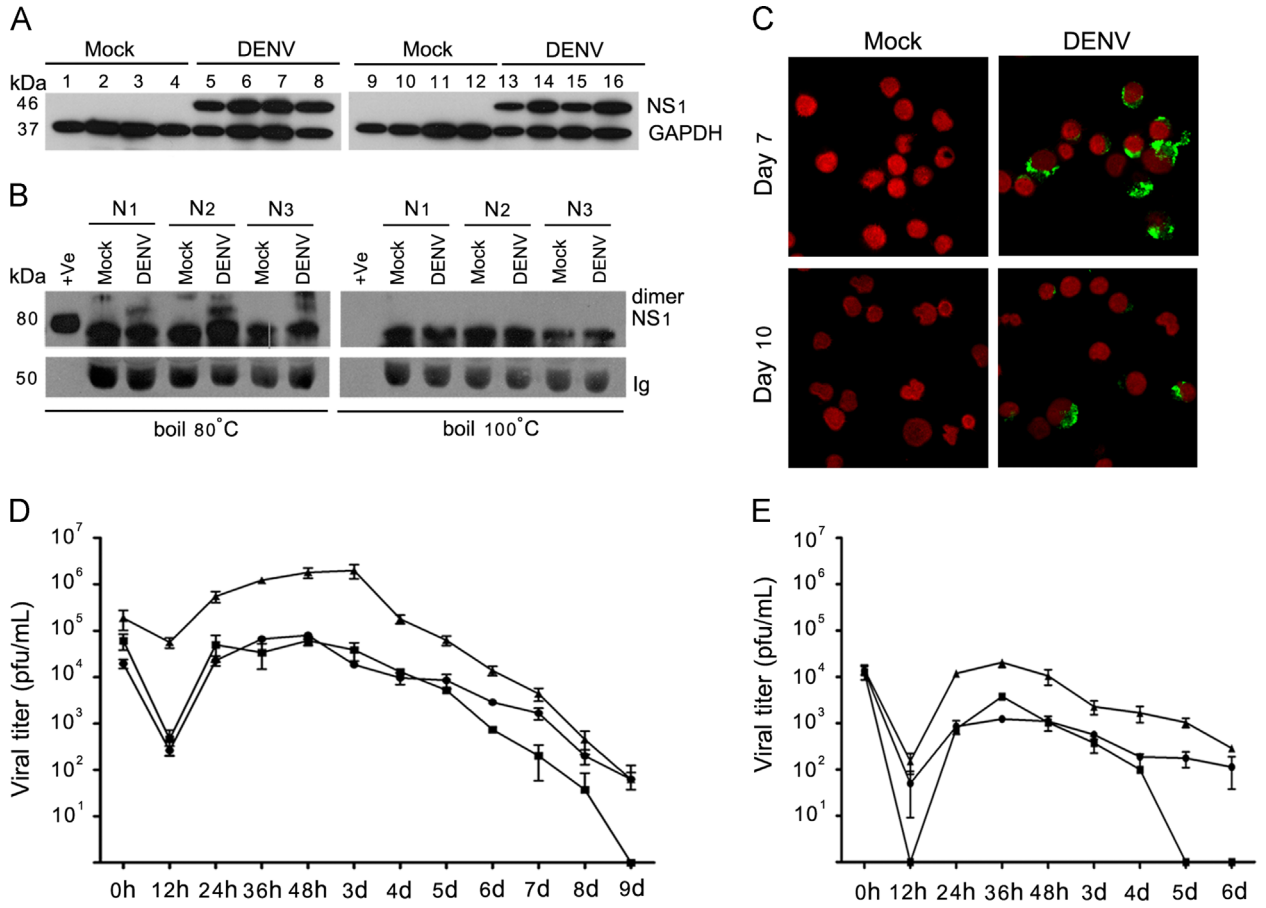

Fig. 2. DENV-2 replication and production in erythroid precursor cells. (A) Erythroid committed precursor cells from normal control (lanes 1, 5, 9, 13), Hb E trait (lanes 2, 6, 10, 14), β-thalassemia trait (lanes 3, 7, 11, 15) and α-thalassemia-1 trait (lanes 4, 8, 12, 16) were either infected (DENV) or mock infected (Mock) on day 7 and day 10 and analyzed after 48 h by western blotting for expression of DENV NS1 and GAPDH in a duplex analysis. (B) Supernatants were collected from day 7 erythroid precursor cells of three normal controls (N1, N2 and N3) at 48 after mock or DENV-2 infection. Protein was heated at either 80 °C or 100 °C and analyzed by western blot for detection secreted DENV NS1. DENV-2 infected erythroid cells were used as a positive control and heavy chain immunoglobulin (Ig) was used as an internal control. (C) Day 7 and day 10 normal erythroid committed precursor cells were either infected (DENV) or mock infected (Mock) and at 48 h.p.i. were stained with a mouse monoclonal anti-DENV NS1 protein antibody followed by a FITC-conjugated secondary antibody (green) and with To-Pro-3 iodide (red) as a nuclear counterstain. Cells were observed under a confocal microscope. Representative merged images are shown. (D and E) DENV production as assessed by standard plaque assay from day 7 (D) and day 10 (E) erythroid committed precursor cells after infection with DENV-2. Virus titer in the supernatant was detrmined at the times shown. Normal control 1 (solid circle), normal control 2 (solid square) and normal control 3 (solid triangle). Error bars represent SD in both panels.

differentiation, cell number and cell apoptosis. While no difference in differentiation was seen in normal control erythroblasts (Fig. 3A), α-thalassemia 1 trait cells showed a significant difference in differentiation in response to infection with trait cells showing a significant reduction in state of differentiation (Fig. 3B). With respect to cell number, both normal control and α-thalassemia 1 trait day 7 infected cells showed a significant deficit in response to DENV infection as compared to uninfected cells (Fig. 3C and D), while only α-thalassemia 1 trait cells showed a deficit on cells infected on day 10 (Fig. 3E and F). Cell apoptosis as assessed by propidium iodide and annexin V staining was increased in both day 7 infected normal control and α-thalassemia 1 trait erythroblasts as compared to mock infected, consistent with the deficit seen in cell number, while no significant difference was observed between day 10 infected and uninfected erythroblasts irrespective of trait status (Fig. 4).

Erythroid differentiation stage and DENV-2 infection

To determine the susceptibility for DENV-2 infection of each erythroid cell stage, day 7 and day 10 erythroid precursor cells were generated from three normal controls, three β-thalassemia trait and 3 Hb E trait carriers and were stained with an anti-DENV NS1 antibody followed by a biotinylated secondary antibody and the chromogenic signal finally developed using DAB. Cells were subsequently counterstained with Wright–Giemsa staining to allow differential counting of cells. A total of 500 cells from each sample were counted and scored for differentiation and the presence of NS1. The results showed that NS1 protein was found only in infected cells (Fig. 5) and that the level of infection correlated with the earlier flow cytometry analysis in that infection rates were higher in day 7 cells as compared to day 10 cells and that day 7 erythroblasts from β-trait and HbE trait carriers were reduced as compared to normal control cells (Table 1). Proerythroblasts and basophilic erythroblasts were markedly more susceptible to DENV-2 infection, and susceptibility was reduced at the polychromatic erythroblast stage and dramatically dropped at orthochromatic erythroblasts (Table 1).

Erythroid committed precursor cells and low passage DENV isolates

As several studies have shown that laboratory adapted DENVs can show behavior distinct from that of low passage DENV isolates (Chaichana et al., 2014; Diamond et al., 2000; Edgil et al., 2003), normal control day 7 erythroid committed precursor cells were mock infected or infected with DENV-2 high passage (16681) as used in the previous experiments and a low passage DENV-2 isolate

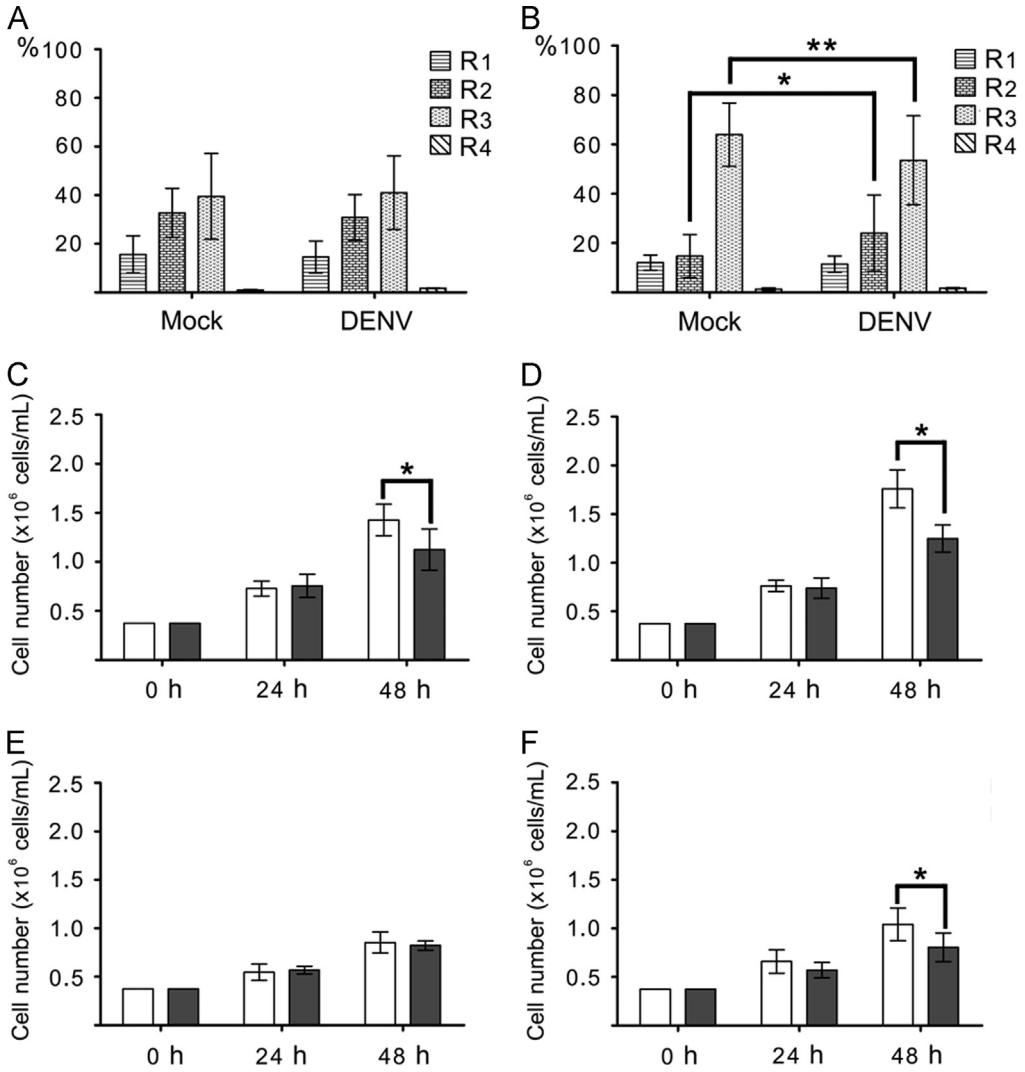

Fig. 3. The effect of DENV-2 infection on cell differentiation and cell number. Differentiation of infected (DENV) and mock infected (Mock) day 7 normal control (A) and α-thalassemia-1 trait (B) erythroid committed precursor cells at 48 h p.i. was assessed by flow cytometry after staining with antibodies directed against CD 71 and glycophorin A. Regions were gated as described previously and data from three experiments was plotted as histograms. (C–F) Infected (solid bars) and uninfected (open bars) day 7 (C and D) and day 10 (E and F) erythroid committed precursor cells from normal controls (C and E) and α-thalassemia 1 trait (D and F) were evaluated for cell number by trypan blue staining at times indicated. Error bars represent SD (*: $p < 0.05$; ** $p < 0.01$).

and a low passage DENV-4 isolate and samples were analyzed by flow cytometry to determine percentage infection. Results showed high levels of infection with the laboratory adapted virus, but undetectable levels of infection with the two low passage isolates (Fig. 6A). To determine more accurately whether any cells had been infected by the low passage isolates, the normal control cells were stained with an anti-DENV NS1 antibody and counterstained with Wright–Giemsa staining as described previously. While infected cells were clearly evident when the infecting virus was the high passage, laboratory adapted strain, no cells showing a similar staining pattern were detected with the low passage strains. However rare scattered cells were positive for NS1 protein, but the morphology suggested that these cells were either dead or dying cells. In this analysis cells were counted before being cytospun onto the glass slides prior to staining and an apparent deficit was noted in cell number with cells exposed to the low passage isolates.

To determine if there was a high degree of apoptosis ongoing in the low passage isolate infections, day 7 erythroid committed precursor cells from both normal controls and hemoglobin E trait were therefore either mock infected or infected with the high and low passage isolates as in the earlier experiment. Cell death was assessed by propidium iodide and Annexin V staining and subsequent flow cytometry at 48 h.p.i. Results (Fig. 6C and D) showed significantly higher levels of cell death of normal control erythroid committed precursor cells infected with low passage DENV-2 as compared to laboratory adapted DENV-2 (Fig. 6C), and markedly, but not significantly higher levels of apoptosis in cells infected with low passage DENV-4 as compared to laboratory adapted DENV-2. Interestingly, both low passage isolates induced significantly higher levels of apoptosis in erythroid committed precursor cells from hemoglobin E trait as compared to the laboratory adapted virus (Fig. 6D). The level of apoptosis induced by the laboratory adapted DENV-2 was significantly higher in hemoglobin E trait cells than in normal control cells ($p=0.02$) consistent with the earlier observation with α-thalassemia 1 trait as compared to normal control (Fig. 4).

The results from infection of erythroid committed precursor cells with low passage isolates of two different serotypes of rare scattered cells of being positive for NS1, but high levels of cell death are consistent with the induction of bystander cell death. Bystander cell death can be mediated by the induction of cytokines such a tumor necrosis factor alpha (TNF-α) capable of triggering apoptosis in surrounding, uninfected cells through the activation of death receptor mediated apoptosis and activation of caspase 8 (Gnesutta and Minden, 2003). To determine if this was the mechanism behind

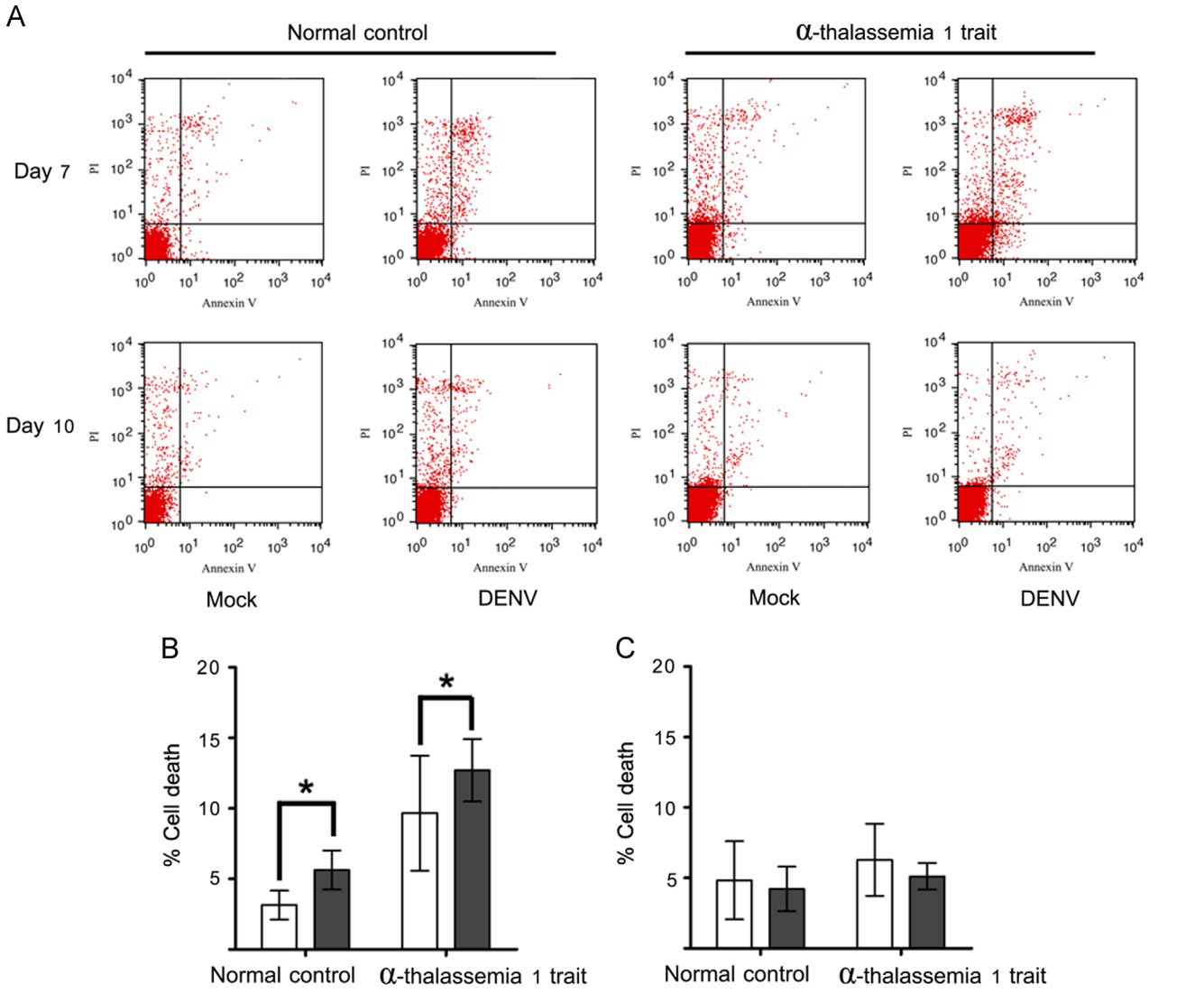

Fig. 4. The effect of DENV-2 infection on cell death. Erythroid committed precursor cells from normal controls and α-thalassemia 1 traits were evaluated for annexin V and propidium iodide staining by flow cytometry. Representative flow cytometry density plots are shown (A) while histograms (B and C) show analysis of three independent experiments. Infected (solid bars) and uninfected (open bars) day 7 (B) and day 10 (C). Error bars represent SD (*; $p < 0.05$).

the high levels of cell death seen in response to infection with low passage isolates, erythroid committed precursor cells from normal controls were infected with low passage DENV-4 in the presence or absence of either an inhibitor of caspase 8 or an antibody directed against tumor necrosis factor receptor 1 (TNF-R1), and at 48 h post infection both the level of apoptosis and the cell number were determined. Pre-treatment of cells with a caspase 8 inhibitor both significantly reduced the level of apoptosis (Fig. 6E) and significantly increased the cell number (Fig. 6F) as compared to non-treated cells. Similarly, post treatment of infected cells with an antibody directed against TNF-R1 significantly reduced the level of apoptosis (Fig. 6G) and significantly increased the cell number (Fig. 6H) as compared to untreated cells.

Discussion

Infection with DENV is a significant public health problem in many tropical and sub-tropical countries of the world and it was recently estimated that there were nearly 100 million apparent dengue infections in 2010, together with some 300 million in apparent infections (Bhatt et al., 2013). To date however there remains no commercially available vaccine and currently there is no specific antiviral drug to treat dengue. Dengue presents with a wide range of clinical symptoms, and while the disease normally resolves itself in a few days, it can be associated with life threatening complications (Gubler, 1998).

The target cells of dengue infection remain relatively poorly characterized, but several cells of the myeloid lineage including dendritic cells (Wu et al., 2000), monocytes (Scott et al., 1980), megakaryocytes (Noisakran et al., 2012) and erythroid progenitor cells (Nakao et al., 1989) have been identified as being targets of dengue infection. This study confirmed the susceptibility of erythroid progenitor cells to DENV infection using a high passage laboratory strain of DENV and additionally showed that erythroid committed precursor cells are also susceptible to DENV infection with the same strain. The study showed that DENV infection of these cells was specific, as we failed to detect infection of these cells with another mosquito borne virus, namely CHIKV an *Alphavirus* of the family *Togaviridae*. Interestingly, the data showed that maximum susceptibility was observed on day 7 of differentiation, in which the majority of cells are predominantly basophilic erythroblasts. HSCs cultured for 4 days (erythroid progenitor) and 10 days (polychromatophilic erythroblasts and orthochromatic erythroblasts) showed much lower

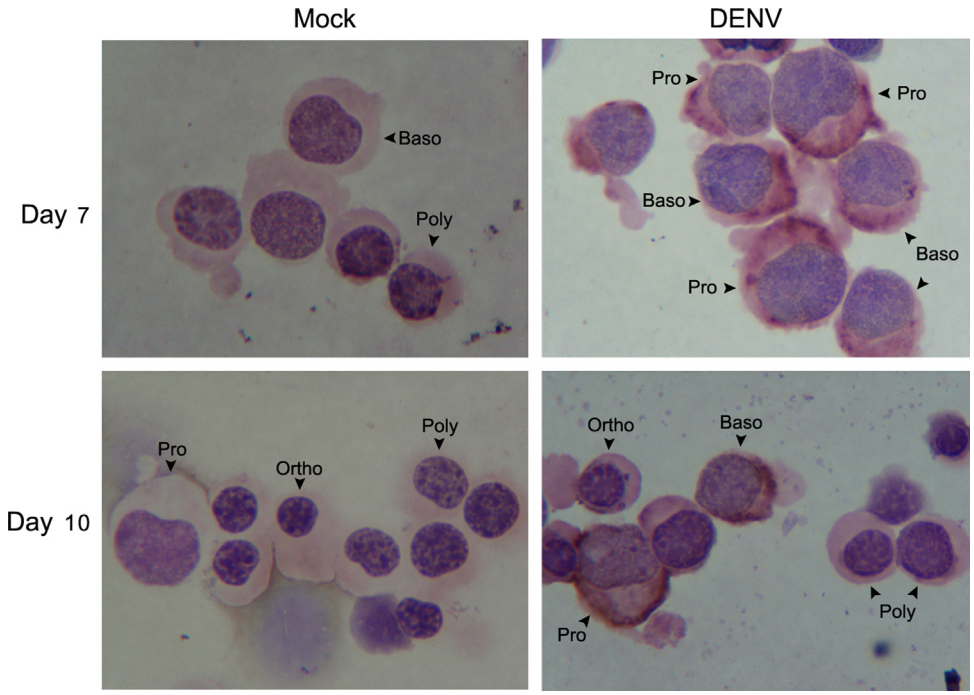

Fig. 5. DENV infection and erythroid stage. Representative fields of DENV infected and mock infected day 7 and day 10 erythroblasts after immunochromogenic NS1 staining and counter staining with Wright–Giemsa stain. Cells were observed under a bright field microscope and DENV NS1 presents as a brown color in the cytoplasm. Identified cells are indicated as proerythroblast (Pro), basophilic erythroblast (Baso), polychromatic erythroblast (Poly) and orthochromatic erythroblast (Ortho).

Table 1
Immunocytochemical staining.

Sample	Value	Cell stage				% NS1 positive
		Pro	Baso	Poly	Ortho	
Day 7 normal control	% stage	5.1	25.1	65.8	4.0	79.7
	% NS1	100.0	99.6	74.4	3.7	
Day 7 β-Thalassemia trait	% stage	1.7	14.5	76.7	7.0	49.7
	% NS1	89.9	87.4	55.2	0.0	
Day 7 Hb E trait	% stage	2.2	10.7	73.5	13.6	51.3
	% NS1	100.0	100.0	45.6	0.0	
Day 10 normal control	% stage	1.0	4.8	61.6	32.6	24.3
	% NS1	100.0	100.0	44.2	0.0	
Day 10 β-Thalassemia trait	% stage	0.1	5.1	68.2	31.4	32.0
	% NS1	100.0	96.4	40.1	0.0	
Day 10 Hb E trait	% stage	0.1	0.7	52.2	47.0	12.3
	% NS1	100.0	100.0	27.5	0.5	

Day 7 and day 10 erythroid committed precursor cells from three normal controls, three β-thalassemia trait and 3 Hb E trait carriers were stained with anti-DENV NS1 and counterstained by Wright–Giemsa staining. The erythroid cell stage proerythroblast (Pro), basophilic erythroblast (Baso), polychromatic erythroblast (Poly) and orthochromatic erythroblast (Ortho) was determined by differential counting and DENV-NS1 positive cells were identified and counted for each stage. Data is shown as the mean of 500 (day 7) or a minimum of 300 (day 10) cells per sample.

rates of infection, suggesting that infection may be mediated by a receptor protein that is transiently expressed around the basophilic erythroblast stage. Infection of erythroid committed precursor cells only occurs through direct infection, and we found no evidence of antibody dependent enhancement of infection as occurs with monocytic cells (Halstead et al., 1980) which corresponds to earlier results that showed that DENV-4 infection of bone marrow progenitor cells was not altered by the presence of antibodies (Nakao et al., 1989).

Infection of erythroid precursors with a high passage isolate was shown to be productive, with *de novo* virus particles being released from the infected cells and so these cells can contribute to the overall level of viraemia which has been shown to be associated with disease severity (Libraty et al., 2002; Vaughn et al., 2000). However, while HSCs are found in the peripheral blood, erythroid committed precursor cells are seldom found in the blood circulation (Constantino and Bogionis, 2000) and as such these cells are unlikely to drive the spread of the virus around the body as has been proposed for megakaryocytes (Noisakran et al., 2012).

Surprisingly, we found a significant reduction in the susceptibility of erythroid committed precursor cells from carriers of thalassemia traits as compared to normal control cells. The protective effects of various globin gene expression hemoglobinopathies and gene deletions in respect of the protozoan parasite Plasmodium have been well documented, but this is the first report of a similar effect in respect of a flaviviral infection. The mechanism of protection in thalassemia carriers remains unclear. In our system both α and β-globin chains are expressed at low levels on day 7 and show a marked increase in expression level by day 10 (Leecharoenkiat et al., 2011). This coincides with both the maximum susceptibility of these cells, as well as the maximum protection seen in trait erythroid precursor cells which were infected on day 7 and analyzed 48 h later (at day 9). Although there is no definitive evidence that thalassemia traits result in a significantly disordered cell processes, it seems likely that even minor imbalances or disorders in globin synthesis can have widespread cellular consequences. In a recent study on hemoglobin H Constant Spring (Hb H-CS) we showed alteration of both chaperone and chaperonin protein expression (Sriiam et al., 2012) and it is possible that these proteins are also disordered to a lesser extent in thalassemia carriers, which may impact upon DENV susceptibility.

As with previous studies (Chaichana et al., 2014; Diamond et al., 2000) we found markedly different consequences when infection was undertaken with high and low passage viruses. We found undetectable levels of infection of cells when low passage DENVs were used as the infecting virus. This is in contrast to the results of Murgue and colleagues who showed that a prototype DENV-3 isolate (H-87) had no effect on erythroid progenitor cells, while low passage isolates reduced the growth of the same cells. There are a number of significant differences between the two studies, as

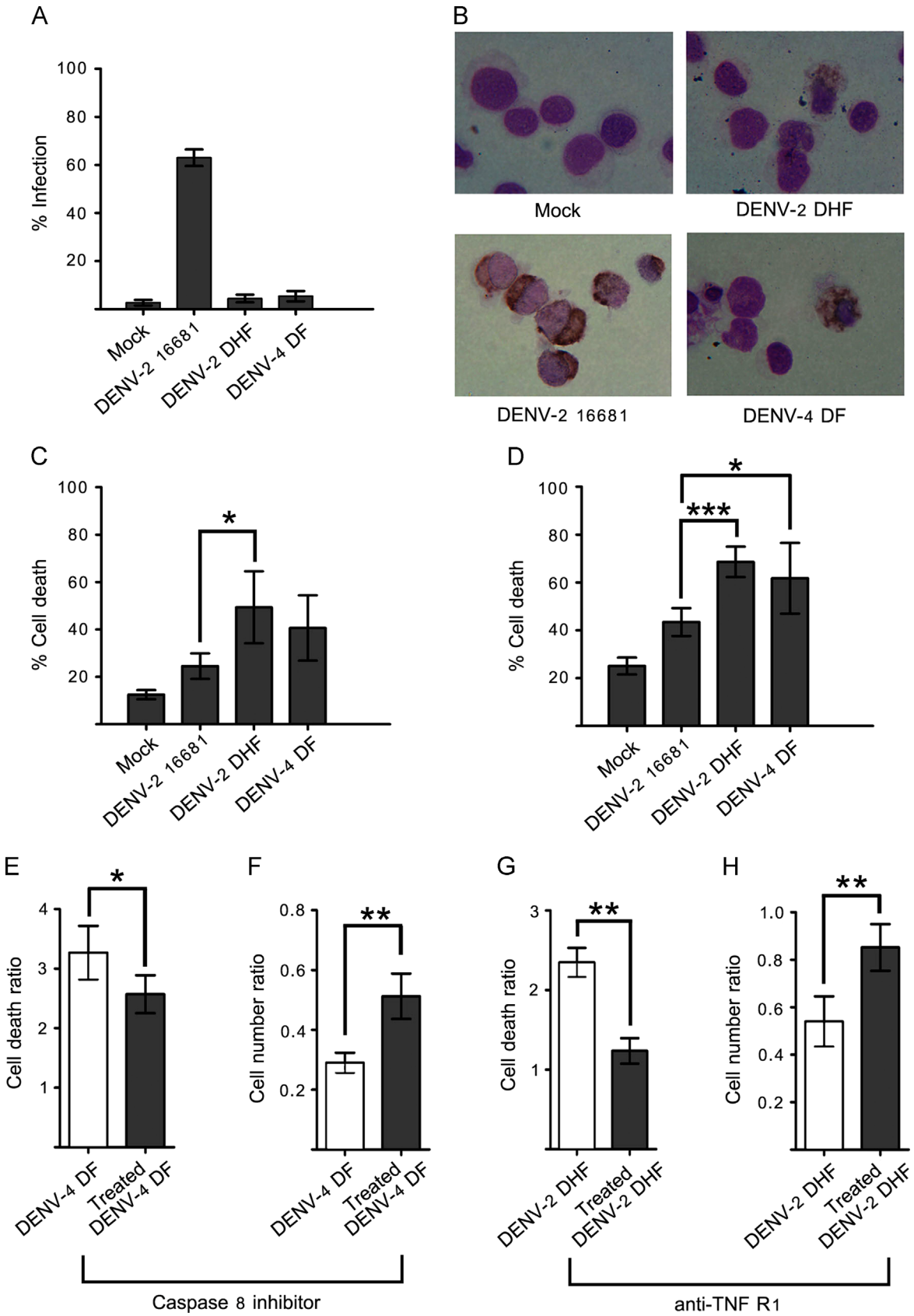

Fig. 6. Clinical isolate DENV infection of erythroid committed precursor cells. The infection of DENV-2 DHF, DENV-4 DF, DENV-2 strain 16681 (positive control), and mock infected cells (negative control) of day 7 erythroid precursor cells from 3 normal controls (A) was assessed by flow cytometry. Both uninfected (Mock) and DENV infected erythroid precursor cells were stained with anti-DENV NS1 and counterstained with Wright–Giemsa staining (B). Cell death of day 7 erythroid precursor cells from 2 normal controls (C) and 3 Hb E traits (D) at 48 h p.i. were evaluated for annexin V and propidium iodide staining by flow cytometry. Cell death (E and G) and cell number (F and H) of DENV-4 DF infection after 10 μM Caspase 8 inhibitor treatment (E and F) and DENV-2 DHF infection after 0.5 μg/mL anti-TNF-R1 treatment (G and H) were determined, all data were normalized against mock and treated mock. Error bars represent SD (*; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$).

Murgue and colleagues (Murgue et al., 1997) investigated erythroid progenitor cells, while our study focused on erythroid committed precursor cells, and our study period was two days post infection while that of Murgue and colleagues (Murgue et al., 1997) was 8 days post infection and the infecting titer was significantly lower in the Murgue and colleagues study (Murgue et al., 1997). It is of particular note that the study by Murgue and colleagues relied solely on cell counts, and provided no direct evidence of DENV infection of these cells (Murgue et al., 1997). While the high infecting titers used in this study may be of some concern, viremia studies in dengue fever (DF) and dengue hemorrhagic fever (DHF) patients have shown median peak viremias of 10Log 9.89 and 10.27 for serum of DF and DHF respectively (Tricou et al., 2011), and as such the titers used in this study are still very far below possible physiological parameters.

While the low passage viruses showed only scattered cells with NS1 positive staining indicative of infection, the low passage isolates induced extremely high levels of cell death as compared to the laboratory adapted virus. Our data suggests that the cell death is induced through activation of capases 8 mediated apoptosis in bystander (uninfected) cells. In this case, although only very few cells maybe infected, bystander apoptosis may be a significant factor in bone marrow suppression. Our results support that the bystander apoptosis was mediated by TNF-α, and high levels of TNF-α have been detected in dengue patients, and levels of TNF-α are believed to be associated with disease severity (Green and Rothman, 2006; Hober et al., 1993; Kittigul et al., 2000).

Bystander apoptosis would serve to dampen infection through removing cells that could otherwise be productively infected adding to the viral load. It is interesting that committed erythroid precursor cells from thalassemia trait carriers both showed a reduced susceptibility to the laboratory adapted virus, as well as markedly increased levels of cell death with low passage isolates as compared to normal control committed erythroid precursor cells. Both of these observations would support that the presence of a thalassemia trait may protect in a small way against severe dengue disease.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.10.004.

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ORIGINAL ARTICLE

Dysregulation of ferroportin gene expression in β^0-thalassemia/Hb E disease

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Abstract During erythropoiesis, iron levels need to be carefully regulated to ensure there is sufficient iron available for hemoglobin synthesis, but that there is no excess to cause damage to the developing erythroblast. Iron influx to the developing erythroblast is controlled by the expression of the transferrin receptor, while iron efflux is regulated by ferroportin (FPN), the sole iron-exporting protein. FPN is encoded through multiple messenger RNAs (mRNAs) some of which contain an iron-responsive element (*variant I* mRNAs) and some of which do not (*variant II* mRNAs). This study sought to investigate the expression of the FPN mRNAs in developing erythroblasts from normal controls and β^0-thalassemia/Hb E patients. While levels of FPN protein were relatively constant, marked reductions of the *variant I* message were seen in erythroblasts from β^0-thalassemia/Hb E patients as compared to normal control cells, particularly in late erythropoiesis. *Variant II* mRNAs were generally increased during erythroid differentiation. No difference was seen in levels of either transferrin or ferritin heavy chain expression. While no difference was observed in labile iron pools under normal culture conditions, erythroblasts from β^0-thalassemia/Hb E patients showed a significantly reduced expression of total FPN message under high iron conditions as compared to normal control erythroblasts. These results are consisted with dysregulation of iron efflux from the maturing erythroblast in β^0-thalassemia/Hb E patients, and this dysregulation possibly contributes to ineffective erythropoiesis seen in these patients.

Keywords β-thalassemia · Iron · Ferroportin · Erythroblasts · Ineffective erythropoiesis

Introduction

β^0-thalassemia/Hb E is an inherited autosomal hematological disorder. The clinical presentation ranges from a mild anemia to severe, blood transfusion-dependent anemia. The disease is caused by defects of the β-globin gene leading to reduced or absent β-globin production [1, 2]. This unbalanced globin chain synthesis allows unpaired α-globin accumulation resulting in ineffective erythropoiesis at the erythroid progenitor stage [3] and mature red cell hemolysis [4]. Consequences of ineffective erythropoiesis can include massive erythroid expansion, extramedullary erythropoiesis, and bone deformation [5].

Iron is an essential component of hemoglobin in red blood cells. Because long-term iron insufficiency causes anemia while excess iron is very toxic to cells, the concentration of iron is tightly controlled. During erythropoiesis, transferrin-bound iron in the serum is taken up by erythroblasts through the transferrin receptor [6]. To ensure the availability of suitable amounts of iron for erythropoiesis, erythroblasts secrete factors that inhibit production of the peptide hormone hepcidin [7]. Hepcidin acts by binding to the sole cellular iron-exporting protein ferroportin [8–10] and promoting its internalization and degradation. Thus, suppression of hepcidin expression results in increased export of iron from iron-storing cells [7]. Somewhat surprisingly, despite the high demand for

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iron during erythropoiesis, erythroblasts also express ferroportin [11, 12]. Ferroportin (FPN1) also known as iron-regulated transporter 1 or Solute carrier family 40 member 1 is encoded by the *SLC40A1* gene [8–10]. This gene is composed of eight exons that give rise to a 571 amino acid protein of approximately 62 kDa [8–10]. Notably, erythroid cells contain two major FPN1 transcripts, an iron-responsive element (IRE)-containing transcript termed FPN1a or *variant I* and non-IRE-containing transcripts FPN1b or *variant II*. Two different forms of *variant II* (A and B) have been reported [11]. However, the protein encoded by all transcripts is identical (Fig. 1).

The expression of FPN1 protein is regulated at several levels. Iron status, heme, other metals, and inflammation have all been reported as factors that control FPN1 transcription [13]. As noted above, the canonical FPN1 *variant I* contains an IRE in the 5'-untranslated region (UTR), and expression of this variant is therefore post-transcriptionally regulated by the iron-responsive element-iron regulatory protein (IRE-IRP) system that responds to iron concentration. Under low-iron conditions, IRP binds to the IRE at 5'-UTR of the IRE-containing FPN1 messenger RNA (mRNA), resulting in inhibition of translation. FPN1 protein expression is therefore diminished, leading to a reduction in iron efflux from cells. Erythroid cells can evade translational repression by the expression of *variant II* FPN1 transcripts which lack an IRE in the 5'-UTR [12].

Previous studies have shown that IRE-containing FPN1 *variant I* mRNA is expressed during early and late erythropoiesis, while *variant II* mRNA is expressed at the middle stage of erythropoiesis [11, 14]. It is believed that coordinated regulation of these transcripts is essential but this has not been rigorously investigated especially under conditions of ineffective erythropoiesis. For this reason, this study sought to determine the expression profile of both *variant I* and *variant II* FPN1 mRNA during erythropoiesis of normal and β^0-thalassemia/Hb E erythroblasts.

Materials and methods

Blood sample collection and erythroid cells culture

This study was approved by the Central Institutional Review Board, Mahidol University. All samples were collected after written informed consent was given. Fifty milliliters of fresh whole blood was obtained from 10 normal controls and 30 mL from 15 mild and 15 severe β^0-thalassemia/Hb E patients. β^0-thalassemia/Hb E patients and controls were diagnosed and screened respectively as previously described [15]. Severity grading (mild, severe) was based on a previously published grading system for β-thalassemia/Hb E disease [16].

CD34+HSCs were isolated from fresh whole blood using the CD34 Microbead Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following the manufacturer's instruction as previously described [15, 17]. Purified CD34+HSCs were grown and induced to proliferate and differentiate in the erythroid lineage in Iscove's modified Dulbecco medium (IMDM, Gibco BRL, Gaithesburg, MD) supplemented with 15 % fetal bovine serum (Gibco BRL), 15 % human AB serum, 10-ng/mL interleukin-3 (IL-3; Promokine, Heidelberg, Germany), 20-ng/mL human stem cell factor (hSCF; Cell signaling technology, Danvers, MA), and 2-U/mL recombinant human erythropoietin (EPO; Janssen-Cilag Ltd, Auckland, New Zealand). Cultured cells were maintained at 37 °C in a 5 % CO_2 incubator. Fresh complete media without IL-3 was freshly prepared and replaced on days 3, 7, and 10, and then cells were continuously incubated until required. This protocol generates cells with greater than 95 % proerythroblast morphology as previously described [15].

RNA extraction and reverse transcriptase PCR

Cultured erythroid cells of five samples of each group: normal control, mild, and severe β^0-thalassemia/Hb E disease, were collected on days 7, 10, and 14 of culture. Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA) following the manufacturer's instructions, and DNA was degraded by using *Dnase I*, RNase-free (Thermo Scientific, Lafayette, CO) at 37 °C for 30 min. Treated RNA samples were re-extracted with TRIzol reagent, and total RNA concentration was measured by using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). After that, cDNA was synthesized using ImProm-II reverse transcriptase (Promega, Madison, WI) in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA). One microgram of total RNA was used as a template, and oligo (dT) (Bio Basic Inc., Amherst, NY) was used as a primer.

Quantitative PCR

Total FPN1, FPN1 *variant I*, TFR1, FtH, and β-actin expression were quantitated using KAPA SYBR FAST qPCR Kit Master Mix (2×) ABI Prism (Kapa Biosystems Inc., Wilmington, MA) in a Mastercycler Realplex (Eppendorf, Hamburg, Germany). complementary DNA (cDNA) was used as a template for amplification using specific primers as follows: FPN1F: 5' GTGGTTTGGTTCGGACAGGTCT3', FPN1R: 5'GATTCAGGACTTGTCTCCGGGAC3', FPN1 Variant IF: 5'CAAACCGCTTCCATAAGGCTTTGC3', FPN1Variant IR: 5'TTCTG CGGCTGCTATCGCTG3', TFR1F: 5'AAATCCGGTGTAGGCACAGC3', TFR1R: 5'TTGCTGGTACCAAGAACCGC3', FtHF: 5'TGCACAAACTGGCCACTGAC3', FtHR: 5'CATGCATGCACTGCCTTGGT3', β-actinF 5'GAAGATGACCCAGATCATGT3' and β-actinR 5'ATCTCTTGCTCGAAGTCCAG3'. Amplification was performed with an initial denaturation at 95 °C for 3 min

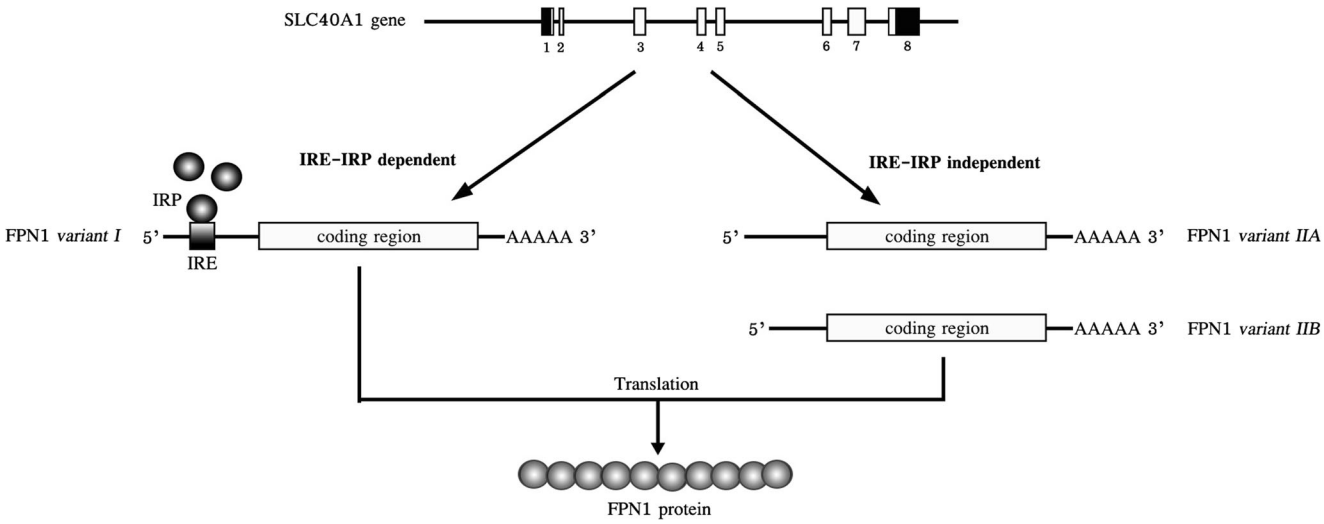

Fig. 1 Ferroportin gene structure and expression. The SLC40A1 gene or ferroportin 1 (FPN1) gene consists of eight exons and transcribes to two major FPN1 transcripts. FPN1 *variant I* contains an iron-responsive element (IRE) in the 5'-untranslated region (5'-UTR), and its expression is regulated by the iron-responsive element-iron regulatory protein (IRE-IRP) system. FPN1 *variant II* is subjected to alternative splicing which gives rise to two isoform mRNAs, FPN1 *variant IIA*, and FPN1 variant *IIB*. The expression of these transcripts is IRE-IRP independent due to the lack of an IRE in the 5'-UTR. All FPN1 transcripts are translated to an identical FPN1 protein

followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60–65 °C for 30 s, and extension at 72 °C for 20 s. The expression of FPN1 was normalized against β-actin by using a $2^{-\Delta Ct}$ calculation, while TFR1 and FtH were normalized against normal control using a $2^{-\Delta\Delta Ct}$ calculation.

Semi-quantitative PCR

The amplification of FPN1 *variant II* was performed using Dream Taq DNA polymerase (Thermo Scientific) and the following primers: FPN1 Variant IIF 5'GGTGTG GCATCTGGTTGGAGTTTC3', FPN1 Variant IIR 5'CCACATCCGATCTCCCCAAGTAG 3'. β-actin primers were as described above. The PCR reaction was carried with initial denaturation at 95 °C for 2 min, followed by 30 cycles (FPN1 *variant II*) or 22 cycles (β-actin) of denaturation at 95 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 40 s, with a final extension step at 72 °C for 7 min. PCR products were separated on 2 % agarose gel and subsequently stained with ethidium bromide. Bands were visualized on a UV lightbox, and intensity was quantitated using Quantity One software (Bio-Rad, Hercules, CA). The expression of *variant II* was normalized against β-actin.

Western blot analysis

Days 7, 10, and 14 erythroid cells were lysed with 2 % CHAPS in 1× TBS. Cell lysates were collected, and protein concentrations were measured by the Bradford assay. Thirty microgram protein lysates were separated by 10 % SDS-PAGE, and proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5 % skim milk in 1× TBS containing 0.1 % Tween-20 for 2 h and subsequently incubated with a rabbit anti-SLC40A1 (FPN1) protein polyclonal antibody (ab85370; Abcam Inc., Cambridge, MA) at a dilution of 1:3000 or a goat anti-actin protein polyclonal antibody (sc-1616; Santa Cruz Biotechnology Inc., Dallas, Texas) at a dilution of 1:4000 overnight at 4 °C. Membranes were washed with 1× TBS containing 0.1 % Tween-20 and incubated with either a goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) at a dilution of 1:5000 for 2 h or a rabbit anti-goat IgG conjugated with HRP at a dilution 1:5000 for 1 h as appropriate. Signals were developed using the Clarity Western ECL substrate (Bio-Rad) and detected on X-ray film.

Colorimetric ferrozine-based labile iron assay

To determine intracellular labile iron levels, 4×10^6 erythroid cells were collected and washed with 1× PBS four times with centrifugation at 400×*g* for 7 min. Cells were subsequently lysed with 300 μl of 50-mM NaOH, and lysates were then continuously shaken for 2 h following which 100 μl of cell lysate was added into 100 μl of 10-mM HCl (diluent of $FeCl_3$ standard solution). The mixture was incubated with 30 μl of iron detection reagent containing 6.5-mM ferrozine (Sigma-Aldrish, Louis, Mo), 2.5-M ammonium acetate, and 1-M ascorbic acid (Sigma-Aldrish) for 16 h. Then, 180 μl of mixture was transferred to a well of a 96-well plate, and absorbance was measured at 562 nm. For this experiment, 1.25–30 ng/mL of $FeCl_3$ in 10-mM HCl was used as a standard. Protein concentration of cell lysate was

measured by the Bradford assay [18]. The labile iron level was calculated as ng/mg protein.

Incubation of erythroid cells with ferric ammonium sulfate

Day 13 erythroid cells of three normal controls and two severe β^0-thalassemia/Hb E patients were incubated with complete culture media containing 0.1 and 1-mM ferric ammonium sulfate. Cells were maintained at 37 °C with 5 % CO_2 for 24 h. Treated cells were collected for determination of intracellular labile iron and FPN1 mRNA expression. Cell death was assessed by counting after staining with trypan blue.

Statistical analysis

The statistical analysis was performed by using PASW statistics 18. The relative expression ratios of FPN1 are presented as mean +/− SEM. Gene expression levels, intracellular labile iron and cell death between normal controls and β^0-thalassemia/Hb E erythroid cells were compared using independent sample *t* tests. Data was considered as a statistical significant at a *p* value of less than 0.05.

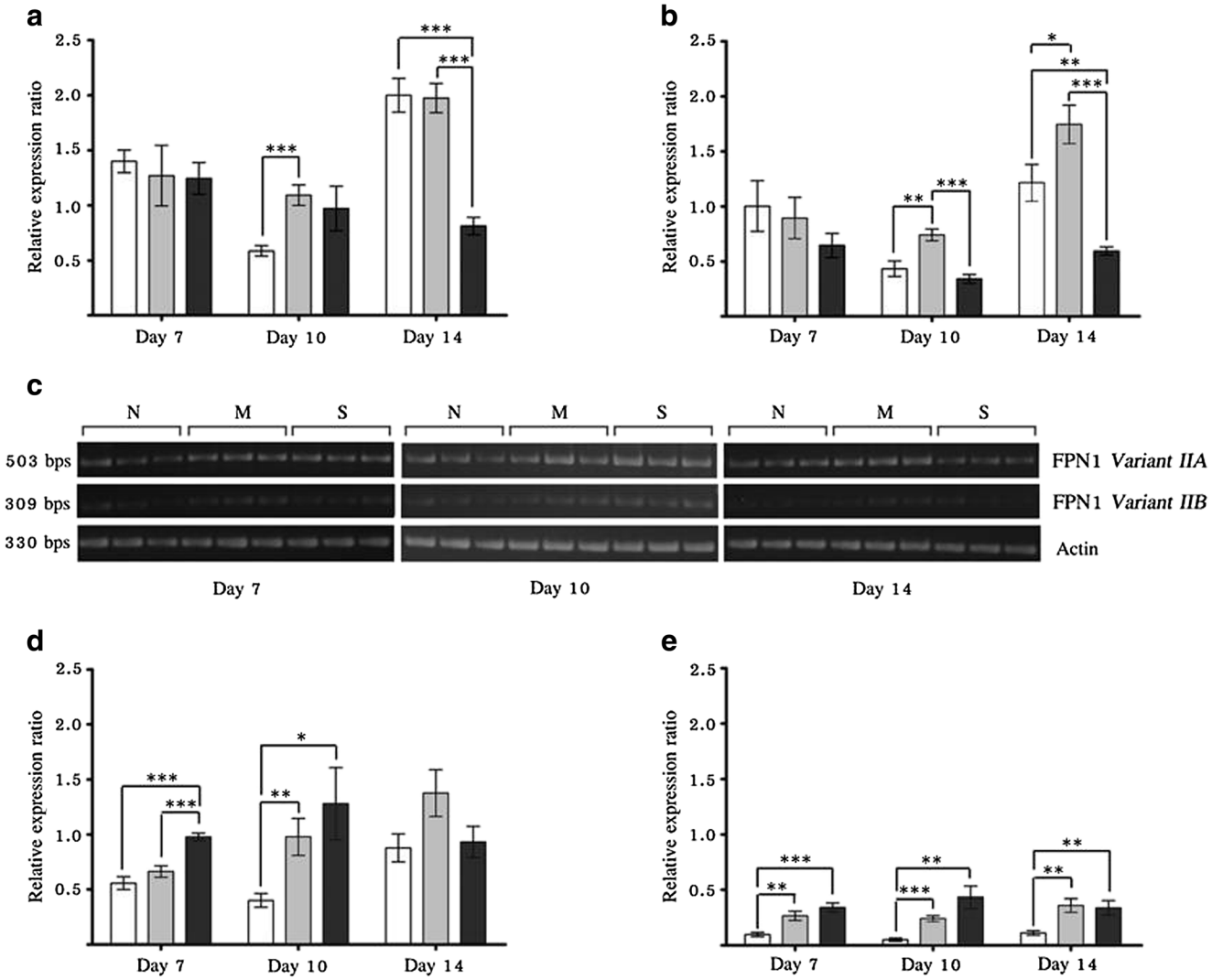

Fig. 2 Ferroportin 1 mRNA expression of erythroid cells. The expression of total ferroportin 1 (FPN1) mRNA expression (**a**), FPN1 *variant I* (**b**) was assessed by quantitative PCR on days 7, 10, and 14 erythroid cells from normal controls (*white bar*), mild (*light gray bar*), and severe β^0-thalassemia/Hb E (*dark gray bar*) patients. The expression of *variant IIA* and *variant IIB* (**c**) was assessed by duplicated semi-quantitative PCR, and their PCR products were separated on 2 % agarose gel normal control (N), mild (M) β^0-thalassemia/Hb E and (S) severe β^0-thalassemia/Hb E. The expression of *variant IIA* (**d**) and *variant IIB* (**e**) was normalized against β-actin, and data is shown as relative expression ratio. *Error bars* represent standard error of the mean (SEM) (*$p<0.05$, **$p<0.01$, and ***$p<0.001$)

Results

FPN1 mRNA expression of erythroid cells

To investigate the expression of FPN1 during normal and ineffective erythropoiesis, CD34+HSCs were isolated from normal control, mild and severe β^0-thalassemia/Hb E patients. The cells were grown under conditions driving erythroid lineage differentiation, although the levels of recombinant human erythropoietin used (2 U/ml) result in accelerated erythropoiesis. Under these conditions, on day 7 of culture, the cell population is predominantly pronormoblast and basophilic normoblast, and the cells mature to later stages under continued culture [19]. In β^0-thalassemia/Hb E, ineffective erythropoiesis results from excess α-globin accumulation which starts to occur when cells initiate globin synthesis at the basophilic normoblast stage (starting around day 7 of culture) [19, 20]. FPN1 mRNA expression was therefore examined in erythroid cells from both normal controls and β^0-thalassemia/Hb E patients on days 7, 10, and 14 of culture. Total FPN1 expression was initially determined by real-time quantitative PCR (Fig. 2a). The result shows that total FPN1 mRNA levels varied during normal erythroid differentiation, with the highest expression on days 7 and 14 of culture, and decreased expression was observed on day 10 of culture. A similar trend was observed in erythroblasts from mild β^0-thalassemia/Hb E patients, although the expression levels of day 10 total FPN1 were significantly higher than normal controls, possibly occurring as a consequence of reduced hemoglobin and hence iron utilization. Interestingly, the total FPN1 expression of severe β^0-thalassemia/Hb E erythroblasts was relatively constant during differentiation and was significantly downregulated on day 14 as compared to erythroblasts from both normal controls and mild β^0-thalassemia/Hb E patients.

Erythroid cells contain 2 FPN1 transcript forms which are translated to the identical FPN1 proteins. To clarify the dysregulation of FPN1 expression, each variant mRNA was specifically examined during cell differentiation. Expression of FPN1 *variant I* for both erythroblasts from normal controls and mild β^0-thalassemia/Hb E patients showed the same alteration in expression as seen for total FPN1, with reduced expression in day 10 as compared to both days 7 and 14. As seen for total FPN1 expression, day 10 erythroblasts from mild β^0-thalassemia/Hb E patients showed significantly higher expression than erythroblasts from normal controls and additionally as compared to erythroblasts from severe β^0-thalassemia/Hb E patients (Fig. 2b). *Variant I* expression in erythroblasts from severe β^0-thalassemia/Hb E patients showed reduced expression on day 10 as compared to days 7 and 14, but the level of expression was significantly reduced on days 10 and 14 as compared to erythroblasts from normal controls and mild β^0-thalassemia/Hb E patients.

Alternative splicing generates two *variant II* mRNAs, *variant IIA*, and *variant IIB*. To distinguish these transcripts, semi-quantitative PCR was used. The PCR products generated (Fig. 2c) were 503 bp (*variant IIA*) and 309 bp (*variant IIB*), and expression was normalized against β-actin (Fig. 2d and e). Surprisingly, expression of *variant IIA* (Fig. 2d) was upregulated in both mild and severe β^0-thalassemia/Hb E erythroblasts as compared to normal control erythroblasts on days 7 and 10 (Fig. 2d). Expression of *variant IIB* (Fig. 2e) was upregulated in erythroblasts from mild and severe β^0-thalassemia/Hb E patients as compared to the cells from normal controls for all days examined.

FPN1 protein expression in erythroid cells

The expression of FPN1 protein was determined in erythroblasts from normal controls and mild and severe β^0-thalassemia/Hb E patients on days 7, 10, and 14 of culture by western blotting, with β-actin expression as a control (Fig. 3a). Normalized expression of FPN1 against β-actin is shown in

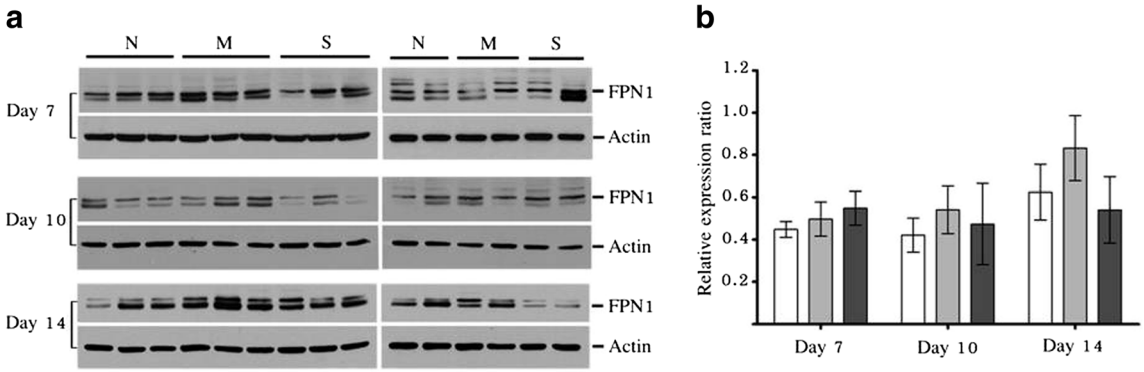

Fig. 3 Ferroportin protein expression of erythroid cells. Ferroportin protein expression on days 7, 10, and 14 of erythroid cells from normal controls (N), mild (M), and severe (S) β^0-thalassemia/Hb E patients was determined by western blot analysis (**a**). The expression was normalized against actin and is shown as a relative expression ratio (**b**). *Error bars* represent standard error of the mean (SEM) (*white bars*; normal control, *light gray bar*; mild β^0-thalassemia/Hb E, and *dark gray bar*; severe β^0-thalassemia/Hb E). No significant differences were observed when comparing between mild or severe patients and normal controls. All *P* values are above 0.05

Fig. 3b. The results show that no significant difference was observed in the expression levels of FPN1 protein as compared between erythroblasts from normal controls and β^0-thalassemia/Hb E patients.

Transferrin receptor 1 and ferritin heavy chain mRNA expression in erythroid cells

To determine whether the observed FPN1 dysregulation was a systemic defect in iron regulation, the expression of two further genes, transferrin receptor 1 (TFR1) which is involved in cellular iron uptake and ferritin heavy chain (FtH) which is involved in cellular iron storage were determined using real-time quantitative PCR. The results (Fig. 4) showed no significant differences in either TFR1 (Fig. 4a) or FtH (Fig. 4b) expression levels, with expression of both genes being relatively constant during differentiation and between erythroblasts from normal controls and from β^0-thalassemia/Hb E patients. However, we note that there was a large variation in TFR1 and FtH expression levels between individuals.

Iron levels and ferroportin expression

To determine labile iron concentrations in maturing erythroblasts, days 10 and 14 erythroblasts were examined using a colorimetric ferrozine-based labile iron assay. Results showed no significant difference in labile iron concentration between normal controls and severe β-thalassemia/Hb E erythroblasts (Fig. 5a and b). We next incubated normal and severe β-thalassemia/Hb E erythroblasts in normal culture medium supplemented with ferric ammonium sulfate (FAS) and observed that incubation with 1-mM FAS significantly increased labile iron concentrations in both normal control and severe β-thalassemia/Hb E erythroblasts (Fig. 5c). This increase in cellular labile iron was not associated with increased cell death (Fig. 5d). Cells were then examined for expression of total FPN1 mRNA (Fig. 5e), the iron-regulated *Variant I* mRNA (Fig. 5f, as well as expression of the non-iron-regulated *variant IIA* (Fig. 5g and h), and *variant IIB* transcripts (Fig. 5g, i). The results clearly show that while normal control erythroblasts significantly upregulated total FPN mRNA in response to increased iron concentrations in the media, erythroblasts from severe β-thalassemia/Hb E erythroblasts showed no increase in total FPN mRNA expression (Fig. 5e). Examination of the individual variants (Fig. 5f–i) showed the increase of FPN mRNA in normal control erythroblasts was due to an increase in expression of *variant I* expression, which did not occur in the severe β-thalassemia/Hb E erythroblasts.

Discussion

During erythropoiesis, erythroid cells require iron at an appropriate level for hemoglobin synthesis. Erythroid cells take up iron through transferrin receptor-mediated endocytosis [21] and are subsequently incorporated with protoporphyrin IX to form heme, a crucial component of hemoglobin [22]. While the majority of iron inside a cell is tightly bound (as for example in hemoglobin), cells also contain a cytosolic labile iron pool [23]. It is unclear whether iron taken up by erythroid cells and destined for heme synthesis pass through the labile iron pool or are targeted directly to the mitochondria in the so-called "kiss and run" pathway [24]. However, studies have shown that the uptake of iron by erythroid cells during development is governed by the level of transferrin receptors expressed, and that iron is taken up at a rate of approximately 36 iron atoms per hour per receptor [25]. Our results showed no significant difference in the level of transferrin receptor mRNA levels between differentiating erythroid cells from normal controls and either mild or severe β^0-thalassemia/

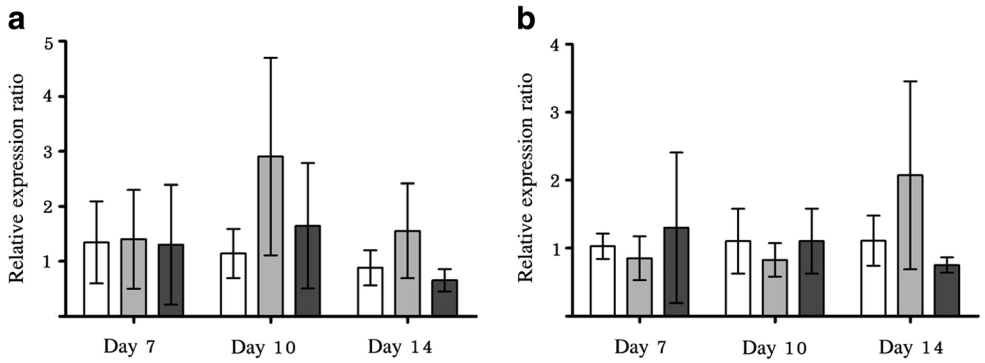

Fig. 4 Transferrin receptor 1 and ferritin heavy-chain mRNA expression of erythroid cells. The expression of transferrin receptor 1 (TFR1) (**a**) and ferritin heavy chain (FtH) (**b**) mRNA expression was assessed by quantitative PCR on days 7, 10, and 14 erythroid cells from normal control (*white bar*), mild β^0-thalassemia/Hb E (*light gray bar*), and severe β^0-thalassemia/Hb E (*dark gray bar*). The expression was normalized against normal control, and data is shown as relative expression ratio. *Error bars* represent standard error of the mean (SEM). No significant differences were observed when comparing between mild or severe patients and normal controls. All *P* values are above 0.05

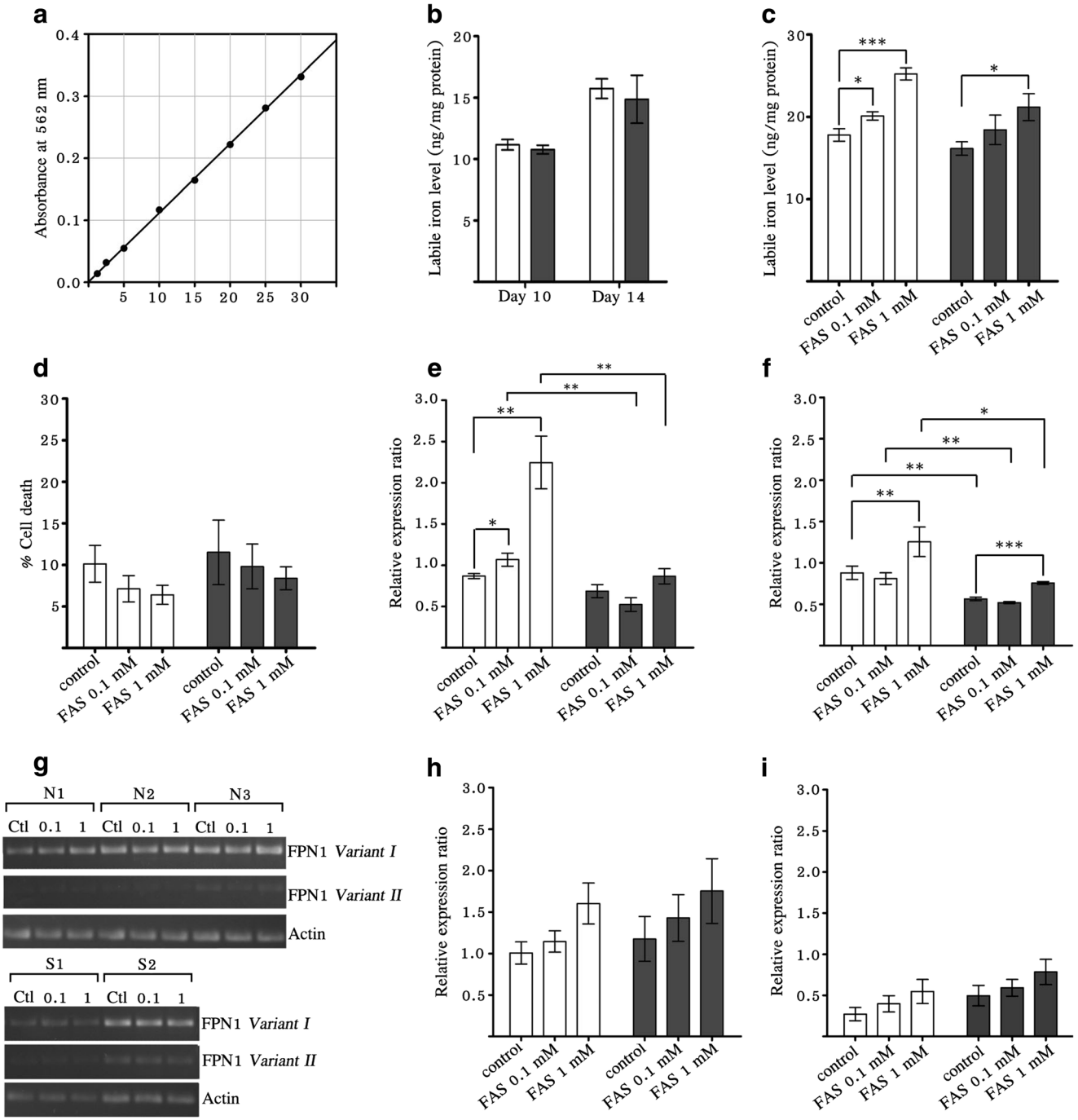

Fig. 5 The effect of increased iron on ferroportin 1 mRNA expression in erythroid cells. (**a**) Labile iron levels were determined by using a colorimetric-ferrozine-based assay with $FeCl_3$ as a standard. The formation of Fe^{2+}-ferrozine complex was measured at 562 nm, and absorbance was plotted as a linear graph with a correlation coefficient of 0.999 (**a**). (**b**) An independent cohort of erythroblasts from five normal controls (*white bars*) and five severe β^0-thalassemia/Hb E patients (*gray bars*) were grown, and labile iron levels were measured on days 10 and day 14. (**c**) To modulate cellular labile iron levels, day 13 erythroid cells of three normal controls (*white bars*) and two severe β^0-thalassemia/Hb E patients (*gray bars*) were incubated with ferric ammonium sulfate (FAS). (**d**) Cell death of cells treated with FAS for 24 h was evaluated through trypan blue exclusion assays. The effect of increased ambient on the expression of total ferroportin 1 (FPN1) mRNA (**e**), FPN1 *variant I* (**f**) was assessed by quantitative RT-PCR, and data is shown after normalization against β-actin. The expression of *variant IIA* and *variant IIB* (**g**) of untreated cells (ctl), FAS 0.1 mM (0.1), and FAS 1 mM (1) was assessed by duplicated semi-quantitative PCR for normal controls (N1, N2, N3) and severe β^0-thalassemia/Hb E erythroid cells (S1, S2). The expression of *variant IIA* (**h**) and *variant IIB* (**i**) mRNAs was normalized against β-actin, and data is shown as a relative expression ratio. *Error bars* represent standard error of the mean (SEM) (*$p<0.05$, **$p<0.01$ and ***$p<0.001$)

Hb E patients, suggesting that the iron take up rate is relatively constant in cells derived from either normal controls or β-thalassemia/Hb E patients. However, it should be noted that results were normalized against β-actin expression, and there is the possibility that expression of this may not be constant during differentiation. While the previous studies have shown that erythroid cells from β-thalassemia patients have a significantly greater labile iron pool as compared to normal controls [23], we were unable to confirm this, and our data shows no significant difference between normal control and severe β^0-thalassemia/ Hb E erythroblasts. We note however that the control erythroblasts in the prior study [23] show a 4–5-fold variation in the relative labile iron pool values, and that both the type of thalassemia and the methodology used was different from that employed in this study. Indeed, given that cells are cultured from HSCs under non-iron overload conditions, it is perhaps unsurprising that we did not see a markedly different labile iron pool when comparing between normal controls and cells from severe β^0-thalassemia/ Hb E. In particular, the prior study [23] used cultures derived from peripheral blood BFUe, which are more mature progenitors than the hematopoietic stem cells (HSC) used in the present study. If conditions of in vivo iron overload affect LIP content during differentiation in culture, it is possible that cells derived from BFUe would be more affected than cells derived from HSC. However, as shown in a previous study [23], our study showed that both normal control and severe β^0-thalassemia/Hb E erythroblasts showed increased labile iron pools when cultured under conditions of increased exogenous iron, again supporting that import of iron is not defective in severe β-thalassemia/Hb E erythroblasts as compared to control erythroblasts.

In common with iron storage cells such as macrophages and hepatocytes [26], erythroid cells also have an iron export pathway mediated by the protein FPN1 [11, 12], as well as a pathway that is able to export iron in the form of heme [27, 28]. We observed that reduced levels of total FPN1 and FPN *variant I* mRNAs were present in erythroid cells from severe β^0-thalassemia/Hb E patients on day 14 of culture as compared to either cells from normal controls or mild β^0-thalasemia/Hb E patients. It should be noted however that β-thalassemia is characterized by accelerated differentiation [3], and day 14 cultures from β-thalassemia/Hb E patients contain a higher proportion of orthochromatic normoblasts than day 14 cultures from normal controls [19]. However, while both total FPN1 and *variant 1* mRNA are upregulated from days 7 to 14 for both normal controls and mild β-thalassemia/Hb E, they are downregulated in severe β-thalassemia/Hb E, suggesting that differentiation status is not a significant factor.

Normal control erythroblasts showed levels of total FPN1 and FPN *variant I* mRNAs that were markedly higher on days 7 and 14 as compared to day 10, consistent with the previous reports [11, 14]. The reduction in FPN1 and FPN *variant I* expression on day 10 of culture correlates with the increased demand for iron with the major period of hemoglobin synthesis [20], while increased expression on days 7 and 14 would serve to allow the export of potentially toxic iron from these cells when there is no increased requirement for iron. While erythroid cells from mild β^0-thalassemia/Hb E patients were consistent with this pattern of expression, the reduction in total FPN1 on day 10 was significantly less than for normal control erythroblasts, with cells from mild β^0-thalassemia/Hb E patients showing only a small decrease in expression from the day 7 levels. This is consistent with cells from mild β^0-thalassemia/Hb E patients showing dysregulation of ferroportin that is intermediate between cells from normal controls and severe β^0-thalassemia/Hb E patients. In contrast, the pattern of expression of total FPN1 in erythroid cells from severe β^0-thalassemia/Hb E patients was one of a decrease in expression throughout the period examined, with no increase in FPN1 expression on day 14 as was seen with cells from both normal controls and from mild β^0-thalassemia/Hb E patients. Markedly however, differences in expression of both total FPN1 and the iron regulated *variant I* mRNA in β^0-thalassemia/Hb E erythroblasts as compared to normal control erythroblasts occur only from day 10, suggesting that it is the major period of globin synthesis that results in the disordered expression of the iron-regulated *variant I* mRNA, which is reflected in total FPN1 mRNA expression.

It is proposed that expression of the *variant II* messages predominantly occurs during the intermediate stage of erythroid differentiation, although our results showed relatively robust expression of *variant IIA* at all stages examined. *Variant IIB* expression was markedly reduced in comparison to *variant IIA*. Expression of *variant IIA* was higher in erythroid cells from β^0-thalassemia/Hb E patients as compared to normal controls on days 7 and 10, while *variant IIB* was significantly increased in erythroid cells from β^0-thalassemia Hb E patients as compared to normal controls on all days examined. This suggests that thalassemic erythroblasts, particularly in severe cases, may compensate for reduced *variant I* mRNA expression by increasing *variant II* expression with the increased early expression (day 7) suggesting that this is initiated as a consequence of the onset globin synthesis.

FPN1 protein as assessed by western blotting was found to be not significantly different, suggesting that the increased *variant II* expression compensated for the markedly reduced *variant I* expression seen in severe cases. As the *variant II* messages do not contain an IRE, these cells will be less sensitive to iron overloading, which could result in the increased labile iron pools seen in β-thalassemia erythroblasts in some studies [23]. The cellular consequences of iron overload can include increased cell death, consistent with the known pathophysiology of β-thalassemia [3].

When ambient iron in the culture media was increased, both normal and severe β^0-thalassemia/Hb E erythroblasts

showed significantly increased cellular labile iron pools. This was associated with a marked and significant increase in total FPN mRNA in normal control cells, but not in those from severe β^0-thalassemia/Hb E patients. Levels of total FPN were significantly lower in erythroblasts from β^0-thalassemia/Hb E patients, as compared to those from normal controls. This again highlights dysregulation of FPN expression in erythroid cells from severe β^0-thalassemia/Hb E patients. The mechanism by which the cells from severe cases of β^0-thalassemia/Hb E fail to upregulate FPN expression under increased iron conditions remain unclear. It is possible that the excess alpha globin chains [5] inhibit a critical iron-sensing regulatory mechanism, but further investigation will be required.

Under conditions of the highest FAS treatment, the iron-regulated *variant I* mRNA was significantly increased in erythroid cells from severe β^0-thalassemia/Hb E patients and normal controls, but again the level of *variant I* mRNA was significantly lower in erythroid cells from severe β^0-thalassemia/Hb E patients as compared to erythroid cells from normal controls. FPN *variant IIA* and *variant IIB* transcripts showed no significant changes in expression under increased ambient iron conditions. In this series of experiments, the increased iron was present for only a relatively short period of time, but it clearly demonstrates a reduced FPN transcriptional response to higher iron concentrations in the cultured erythroid cells from β^0-thalassemia/Hb E patients as compared to normal controls. The short period of increased iron was selected as studies have shown that prolonged incubation of erythroid cells under conditions of iron overload suppresses erythroid cell differentiation and induces apoptosis [29]. By selecting day 13 cells for study, 24-h incubation coincides with the maximum FPN1 deficit seen in severe β-thalassemia/Hb E patients. In chronically iron overloaded β^0-thalassemia/Hb E patients where the entire differentiation process is undertaken under conditions of iron overload, this failure could rapidly lead to severe cellular consequences, and studies have shown increased non-transferrin-bound iron (NTBI), transferrin saturation, and increased serum ferritin in β-thalassemia/Hb E patients as compared to normal controls [30]. In addition, higher LIP levels have been documented in red blood cells and reticulocytes from thalassemic patients as compared to the levels found in normal controls [23].

Combined, our results suggest that the regulation of iron influx and efflux, which is an important mechanism during erythropoiesis, is dysregulated in β^0-thalassemia/Hb E, and this possibly leads to ineffective erythropoiesis, a hallmark β^0-thalassemia/Hb E erythroblasts. Further studies into the mechanism of efflux of iron from erythroblasts might lead to novel therapies to reduce iron overload and ineffective erythropoiesis in β-thalassemia disease.

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Compliance with ethical standard This study was approved by the Central Institutional Review Board, Mahidol University. All samples were collected after written informed consent was given.

Conflict of interest The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Mitochondrial Changes in β^0-Thalassemia/Hb E Disease

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Abstract

The compound β°-thalassemia/Hb E hemoglobinopathy is characterized by an unusually large range of presentation from essentially asymptomatic to a severe transfusion dependent state. While a number of factors are known that moderate presentation, these factors do not account for the full spectrum of presentation. Mitochondria are subcellular organelles that are pivotal in a number of cellular processes including oxidative phosphorylation and apoptosis. A mitochondrial protein enriched proteome was determined and validated from erythroblasts from normal controls and β°-thalassemia/Hb E patients of different severities. Mitochondria were evaluated through the use of mitotracker staining, analysis of relative mitochondrial genome number and evaluation of mitochondrial gene expression in addition to assay of overall cellular redox status through the use of alamarBlue assays. Fifty differentially regulated mitochondrial proteins were identified. Mitotracker staining revealed significant differences in staining between normal control erythroblasts and those from β°-thalassemia/Hb E patients. Differences in relative mitochondria number and gene expression were seen primarily in day 10 cells. Significant differences were seen in redox status as evaluated by alamarBlue staining in newly isolated CD34+ cells. Mitochondria mediate oxidative phosphorylation and apoptosis, both of which are known to be dysregulated in differentiating erythrocytes from β°-thalassemia/Hb E patients. The evidence presented here suggest that there are inherent differences in these cells as early as the erythroid progenitor cell stage, and that maximum deficit is seen coincident with high levels of globin gene expression.

Introduction

The thalassemias are a diverse group of hematological disorders arising from the inheritance of defective globin genes [1]. The compound β°-thalassemia/Hb E hemoglobinopathy is common

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in Southeast Asia, and is characterized by a wide range of presentation from essentially asymptomatic to a severe, transfusion dependent condition [2]. While a number of factors including the co-inheritance of α-globin hemoglobinopathies and the level of HbF, have been shown to play a role in modulating the presentation of the disease, these factors do not account completely for the range of presentation found for apparently comparable underlying genetic lesions [2], suggesting that other factors remain to be found that modulate the presentation of the disease.

The primary characteristic of β°-thalassemia/ Hb E is anemia of a variable severity. The anemia arises from a combination of ineffective erythropoeisis and increased hemolysis of the mature red blood cells [3, 4]. Ineffective erythropoiesis occurs as a consequence of apoptosis occurring at the polychromatophilic normoblast stage of erythropoiesis [3], and the small proportion of cells that do mature to red blood cells undergo increased hemolysis as a consequence of the deposition of unpaired α-globin chains in the cells [4]. As a consequence of the anemia, levels of erythropoietin are increased [5] leading to expansion of the erythroid mass, but because of ineffective erythropoiesis this does not result in significant alleviation of the anemic state. Previous studies have noted that cultured erythroid progenitor cells from β°-thalassemia/ Hb E patients show increased cell expansion and increased differentiation as compared to erythroid progenitor cells from normal controls [6]. The reasons for the increased expansion of β°-thalassemia/Hb E erythroid precursor cells remain unclear. It is possible that this results from some form of conditioning in which the progenitor cells from thalassemia patients are primed to undergo increased expansion as a consequence of the higher levels of EPO in the patients from which the cells are taken, or alternatively the increased expansion could reflect an inherent difference in the cells.

In a previous study we undertook a proteomic analysis of erythroid precursor cells from both normal controls and β°-thalassemia/Hb E patients [7]. That study showed increased levels of a number of proteins in β°-thalassemia/ Hb E erythroid precursor cells, of which the largest single class was proteins involved in glycolysis and the tricarboxylic acid (TCA) cycle. We additionally demonstrated increased levels of oxidative phosphorylation in these cells [7]. The TCA cycle and oxidative phosphorylation occur in the mitochondria, a maternally inherited subcellular organelle [8]. Studies have shown that mitochondria vary greatly in their numbers and in their activity depending upon the energy requirements of the cell [9]. Mitochondria have their own genetic material which encodes for some 37 genes of which code for 2 ribosomal RNAs (rRNAs), 22 for transfer RNAs (tRNAs) and 13 for polypeptides which are core protein subunits of the oxidative phosphorylation system (comprehensively reviewed by Friedman and Nunnari [10]). The majority of proteins involved with the TCA cycle and oxidative phosphorylation are therefore genes encoded in the cellular genome, and not the mitochondrial genome, and these proteins determine metabolic function and activity of the mitochondria [11]. Given the association with the TCA cycle and oxidative phosphorylation, processes that we have shown disordered in β-thalassemia/ Hb E erythroid cells [7], this study sought to take a more detailed look at mitochondria and their relationship with β-thalassemia/Hb E disease.

Materials and Methods

Patients, Sample Collection and Erythroid Cell Culture

This study was approved by the Ethical committee, Mahidol University Institutional Review Board (IRB 2009/038.0202). Written informed consent was obtained from participants before sample collection. Patients were identified, disease severity graded and controls were screened as previously [6]. Fifty ml of peripheral blood was taken from healthy controls and 25 ml of peripheral blood was taken from patients. CD34+ cells were isolated from peripheral blood

and cultured in supplemented Iscove's modified Dulbecco medium as previously described [6]. Cell numbers were determined by trypan blue staining using a hemocytometer.

Protein Preparation and GeLC-MS/MS

Mitochondria were purified from day 10 erythrocytes from 5 normal controls, 5 mild and 5 severe β°-thalassemia/Hb E patients using magnetically labeled anti-TOM22 microbeads (Miltenyi Biotech, Auburn, CA) and separation on an LS Column placed in a MidiMACS Separator (Miltenyi Biotech) according to the manufacturers protocol. Mitochondria preparations were lysed with 0.5% SDS and sonicated for 5 min twice. Proteins from each group were pooled and subsequently analyzed in duplicate. GeLC-MS/MS and protein identification was undertaken exactly as previously described [12], except that the Mitoproteome database [13] was searched in addition to the NCBI database.

Western Blotting

Western blotting was undertaken essentially as described elsewhere [7]. A total of 30μg of proteins were run per lane on 12% SDS-PAGE gels. Primary antibodies used included a 1:1000 dilution of a rabbit polyclonal anti-Prohibitin 2 (Santa Cruz Biotechnology, Santa Cruz, CA) antibody and a 1:1000 dilution of a rabbit polyclonal anti-HSP60 antibody (Santa Cruz Biotechnology).

Mitotracker Staining

Erythroid precursor cells were incubated with 500 nM MitoTracker Red CMX Ros (MTR, Molecular Probes, Eugene, OR) for 15 min following which cells washed with PBS-IFA (0.15M NaCl, 0.05M NaH_2PO_4, 0.05M Na_2HPO_4, pH 7.4), cytocentrifuged onto glass slides, fixed with 4% paraformaldehyde, permeablilized with 0.3% tritonX/PBS and counterstained with DAPI (Merck Millipore, Temecula, CA) before visualization under a confocal microscope. For flow cytometry cells were stained with mitotracker as above, washed with PBS-IFA and analyzed directly by flow cytometry (BD, FACSCalibur) using the CELLQuest software (BD Biosciences).

AlamarBlue Assay

AlamarBlue (Thermo Fisher Scientific, Waltham, MA) assays were undertaken according to the manufacturers' protocol on 1 x 10^4 cells.

Quantitative Real-Time PCR

At least 1x10^6 cells were collected by centrifugation, washed with 1x ice-cold PBS and re-centrifuged. The cell pellet was resuspend in 1 ml of TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) and DNA and RNA prepared according to the manufacturer's instructions. mRNA was reverse transcribed using random primers and ImpromII™ reverse transcriptase (Promega, Madison, WI). Real-time PCR amplification (first strand cDNA and DNA) was performed in 20μL containing 2X Kapa SYBR® FastMaster Mix (Kapa Biosystems, Inc., Wilmington, MA), 300 nM each primer (Table A in S1 File) and 5ng (cDNA) or 50ng (DNA) template. Primers and cycle conditions are given in Table A in S1 File. mRNA expression data was normalized to β-actin, while mitochondrial DNA copy number was normalized to ferroportin 1A.

Statistical Analysis

Statistical analysis was performed using the PASW statistics 18 (SPSS Inc. Chicago, IL). And all data was compared using independent sample *t*-tests. Data was considered as a statistical significant at a *p* value of less than 0.05.

Results

Mitochondria Enriched Proteome Analysis

To investigate the profiles of mitochondria associated proteins in β°-thalassemia/Hb E patients as compared to normal controls, mitochondria enriched proteins were obtained from day 10 erythroblasts of five normal controls, five severe and five mild β°-thalassaemia/Hb E patients, using anti-TOM22 microbeads to purify mitochondria followed by protein extraction. The protocol was shown to significantly enrich for mitochondrial proteins as shown by Western blotting for NADH:ubiquinone oxidoreductase subunit A9 (NDUFA9; Fig A in S1 File). The proteins were analyzed by a gel-enhanced liquid chromatography tandem mass spectroscopy (GeLC-MS/MS) [14]. Original gels are shown in Fig B in S1 File. Resultant analysis of the generated spectra identified 4392 peptides corresponding to 1837 proteins, of which some 1428 were not differentially expressed between samples (Fig 1a). Ontological analysis of these proteins for biological process and molecular function is shown in Fig 1c and 1d. The spectra were subsequently screened against the Mitoproteome database on 10th and 19th June 2013 which identified some 288 mitochondrial proteins, representing approximately 40% of the database annotated mitochondrial proteome. Of these 288 proteins, three proteins showed significantly increased expression in mild β°-thalassemia/Hb E patients only, while a further five proteins showed significantly increased expression in severe β°-thalassemia/Hb E patients only. A total of forty-two proteins were significantly up-regulated in both mild and severe β°-thalassemia/Hb E patients as compared to normal controls (Fig 1b and Table B in S1 File). The heat map is shown in Fig C in S1 File. Interestingly, no protein was detected as showing down-regulation in β°-thalassemia/Hb E erythroblasts as compared to normal control erythroblasts. To validate the proteomic data, western blot analysis of two proteins identified as differentially expressed was undertaken on samples from an independent cohort of patients and controls. The proteins selected, heat shock protein 60 (hsp60) and prohibitin2 are both well characterized mitochondrial chaperone proteins and both have been shown to have additional, albeit predominantly antagonistic, roles in the regulation of apoptosis (reviewed in [15]). As the proteome data showed discordant expression of these two proteins (hsp60 up in severe and prohibitin 2 down in severe) despite their similar chaperone functions their expression profile was considered to be important to validate. Both proteins showed results consistent with the proteome data (Fig 2).

The 50 proteins identified as being significantly up-regulated in β°-thalassemia/Hb E patients belonged to a number of cellular processed including oxidative phosphorylation and energy metabolism (Table B in S1 File). Ontological analysis using the GoCat software [16] showed that most of the differentially expressed proteins were involved in cellular processes (30%), regulation (17%) and metabolic processes (8%) as shown in Fig 1e. Functional categorization of the significantly differentially expressed proteins indicated up to 51% of the proteins were characterized as binding while 37% were characterized as having catalytic activity (Fig 1f).

Mitochondria in β°-Thalassemia/Hb E

Mature red blood cells do not contain mitochondria [17] and it is well established that mitochondria are lost by the process termed mitophagy (reviewed by Mortensen and colleagues [18]), and it is generally believed that mitochondria are lost during reticulocyte maturation

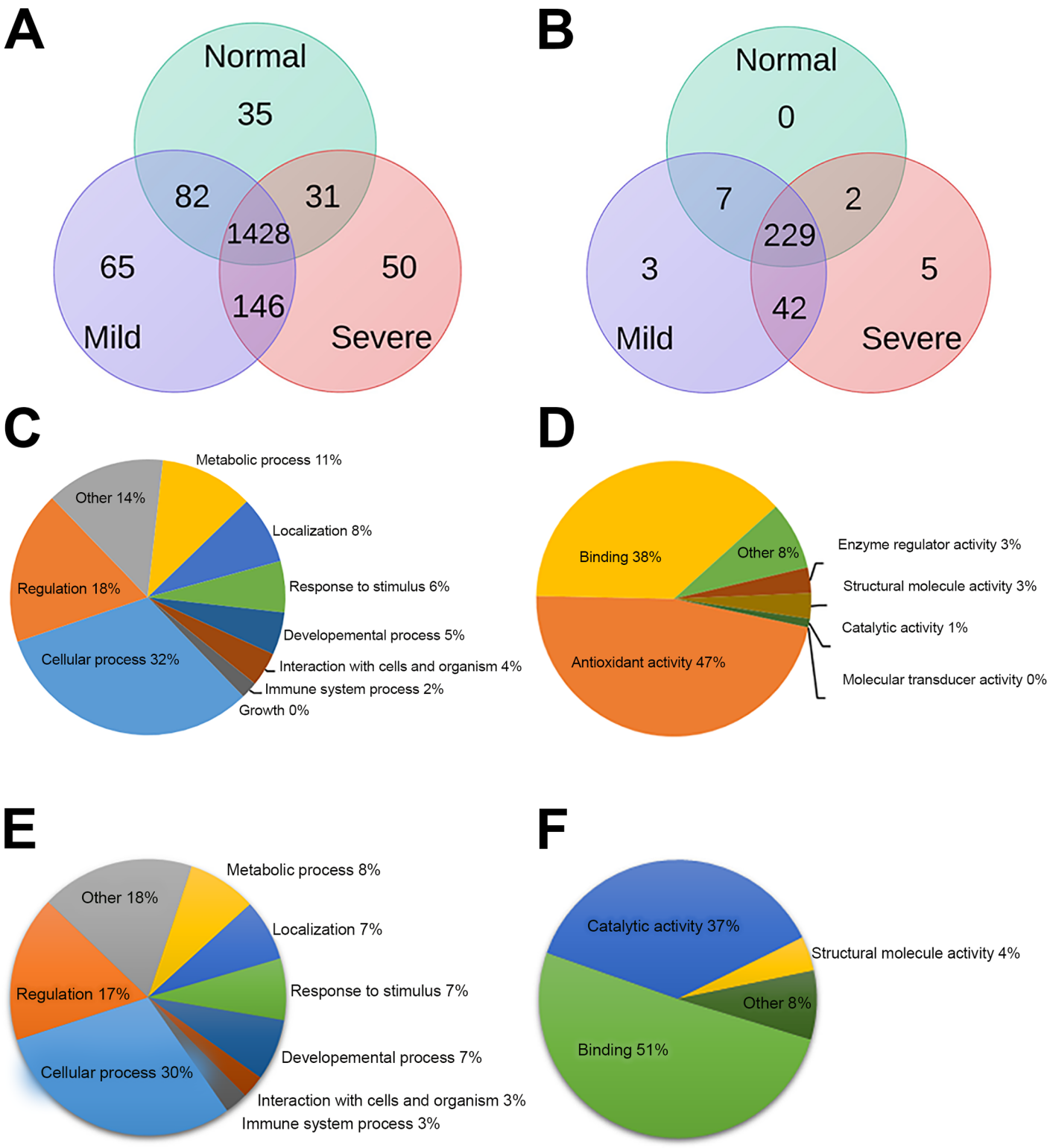

Fig 1. The mitochondrial protein enriched proteome of erythroid cells. Mitochondrial protein enriched preparations from day 10 erythroid cells from normal controls and β°-thalassemia/Hb E patients (mild and severe) were subjected to GelC-MS/MS analysis. Venn diagrams of (a) total proteins identified and (b) mitochondrial proteins as identified by the Mitoproteome database and GoCat ontological analysis of (c) cellular processes and (d) functional categorization of the identified mitochondrial proteins and GoCat ontological analysis of (e) cellular processes and (f) functional categorization of the identified differentially regulated proteins.

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A

ID Details	Peptide Intensity					
	Normal1	Normal2	Mild1	Mild2	Severe1	Severe2
HSP60 [Homo sapiens]	0.0	0.0	0.0	0.0	6.1	7.7
Prohibitin2 [Homo sapiens]	6.9	9.8	6.4	9.9	0.0	0.0

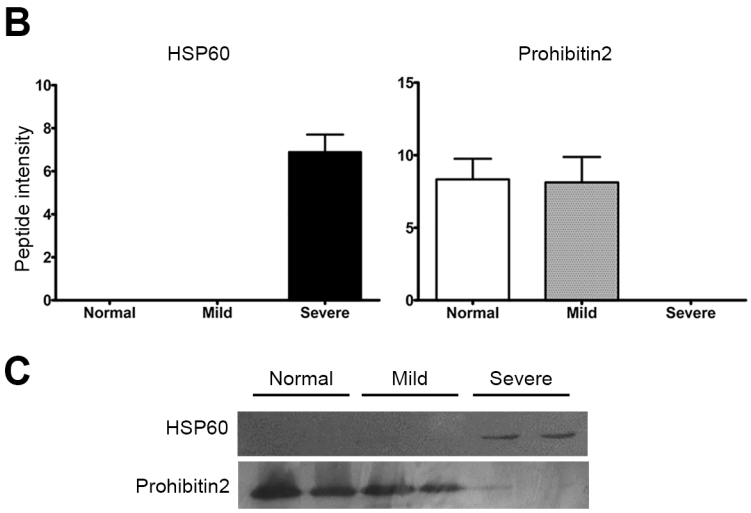

Fig 2. Validation of proteome data. Based on the proteome data, two differentially regulated mitochondrial proteins (hsp60 and prohibitin 2) were selected for validation in an independent cohort of controls and patients. Raw proteome data reads (a) and quantitation (b) are shown together with (c) western blot analysis of the independent cohort. Samples were pooled from 5 individuals and run as duplicate lanes.

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[17]. To directly observe mitochondria during erythroblast differentiation cells on days 7, 10 and 14 were stained with mitotracker and observed under a confocal microscope. Results showed markedly different patterns of staining between cells from normal controls and those from β°-thalassemia/Hb E patients (Fig 3). This was most clearly observed on day 7 of culture whereby the signal in normal control cells was predominantly perinuclear, while in cells from β°-thalassemia/Hb E patients the signal was largely colocalized with the nuclei (Fig 3).

Quantitative analysis of the signal by flow cytometry showed significant differences in the levels of signal between normal control cells and those from β°-thalassemia/Hb E patients (Fig 3). Mitotracker dyes are cationic fluorophores that accumulate in mitochondria as a consequence of the negative mitochondrial membrane potential, and inside the mitochondria the dye forms covalent bonds with proteins and peptides [19]. However, studies have shown that mitotracker dyes preferentially label selected proteins, and while the full list of preferential proteins is not know, studies have shown that hsp60 is a major labeling target of mitotracker dyes [20]. As shown in both the proteome analysis and in the western blotting validation with an independent sample cohort, hsp60 is highly differentially regulated between normal control erythroblasts and erythroblasts from β°-thalassemia/Hb E patients. Thus, results on quantitation of mitochondria using mitotracker need to be interpreted with extreme caution.

To provide an alternative method of quantifying levels of mitochondria, the relative levels of mitochondrial genomes were determined using quantitative PCR, with normalization against the nuclear genome. Results (Fig 3) show equal relative numbers of mitochondria on days 7 and 14, but higher relative levels of mitochondria in cells from β°-thalassemia/Hb E patients

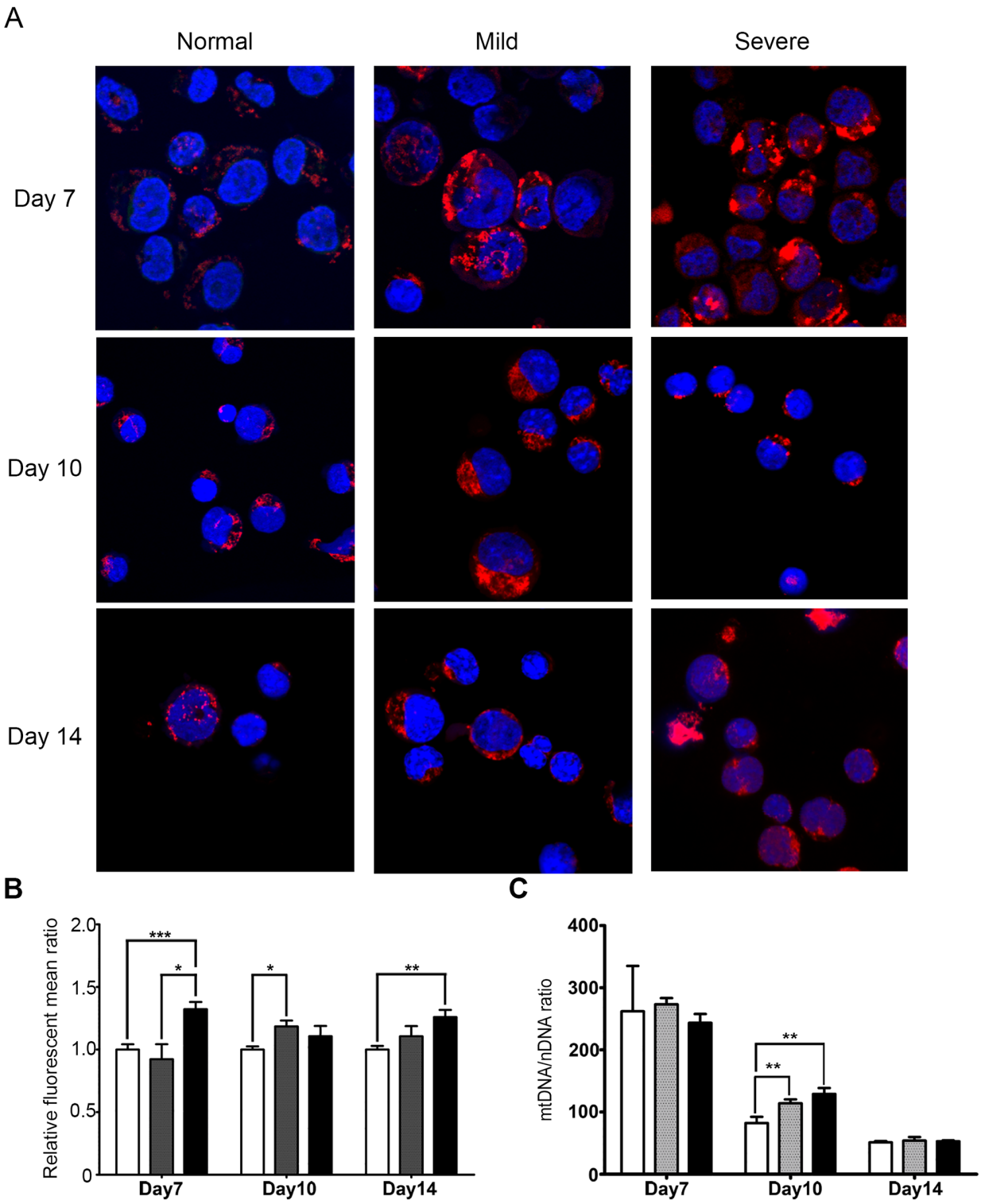

Fig 3. Mitochondria in erythroid precursor cells: quantity. Erythroid precursor cells on days 7, 10 and 14 of culture were (a and b) stained with mitotracker and examined by (a) confocal microscopy or (b) flow cytometry. (a) Representative merged confocal images are shown, and individual fields and merges are shown in Fig D in S1 File. (b) Tabulated flow cytometry data is shown, and individual scatterplots are shown in Fig E in S1 File. (c) Real-time quantitative PCR was used to determine relative mitochondria numbers. Data is normalized to nuclear genome through the ferroportin 1A gene. (b and c) white bars represent normal controls, grey bars are from mild and black bars are from severe β^0-thalassemia/Hb E patients. Error bars show ± S.E.M. * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$.

doi:10.1371/journal.pone.0153831.g003

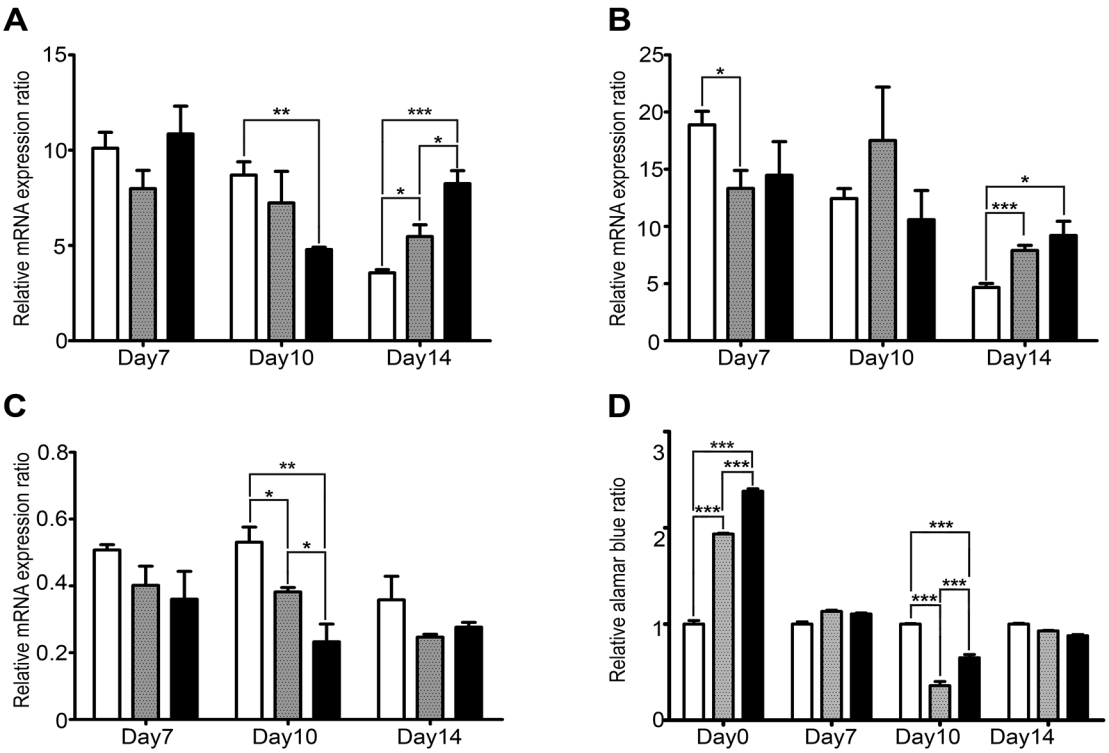

Fig 4. Mitochondria in erythroid precursor cells: activity. Day 7, 10 and 14 erythroid precursor cells from normal controls (white bars) and from mild (grey bars) and severe (black bars) β°-thalassemia/Hb E patients were examined for expression of (a) ATP6, (b) ATP8 or (c) CYTB by real time PCR on days 7, 10 and 14 of culture. (d) A total of 1 x10^4 erythroid precursor cells from normal controls (white bars) and from mild (grey bars) and severe (black bars) β°-thalassemia/Hb E patients were used in alamarBlue assays. Error bars show ± S.E.M. * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$.

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(both mild and severe) on day 10 of culture. Overall, the trend for all samples was consistent with the gradual loss of mitochondria during differentiation, rather than a loss of these organelles only during reticulocyte maturation.

Studies have suggested that mitochondria can be heterogeneous with respect to their activity. We therefore used quantitative PCR to determine relative expression levels of three mitochondrially encoded genes, namely ATP synthase F0 subunits 6 and 8 (ATP6 and ATP8) and mitochondrial cytochrome b (CYTB). Results (Fig 4) showed significant differences during differentiation for all three genes investigated. In particular expression of ATP6 and CTY were reduced in erythroblasts from severe β°-thalassemia patients as compared to normal controls on day 10 of culture. Interestingly both ATP6 and ATP8 were increased in expression in both mild and severe β°-thalassemia as compared to normal controls on day 14 of culture.

Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a commonly used oxidation reduction dye used to determine cell viability available commercially under a number of names including AlamarBlue. The assay measures the conversion of resazurin to resorufin which is mediated by mitochondrial reductases and other enzymes including NAD(P)H: quinine oxidoreductase, and reduction of AlamarBlue may signify impaired cellular metabolism, albeit not mitochondria specific dysfunction [21]. However, the AlamarBlue assay may be interpreted to give a read out of cellular redox status [21]. Constant numbers of erythroid progenitor (day 0 of culture) and erythroid precursor cells (days 7, 10 and 14 of culture) from normal controls and from β°-thalassemia/Hb E patients were therefore assayed by the AlamarBlue assay. Results (Fig 4) clearly show dysregulation in cells from thalassemia patients. In particular,

significantly lower levels are seen on day 10 in cells from thalassemia patients. Interestingly however, cells on day 0 of culture (newly isolated CD34+ cells) show significantly higher levels (for both mild and severe patients) as compared to normal controls (Fig 4).

Discussion

The size of the mitochondrial proteome is still poorly defined. The manually curated MitoProteome database lists 780 proteins as "current", with a further 175 classified as "MS doubtful" and a further 317 as "doubtful". However, it is clear that the mitochondrial proteome is highly variable by tissue [22], and some authors have proposed that there may be as many as 1500 mitochondrial proteins [23]. In our analysis mitochondria were purified through a magnetic bead isolation system, and therefore the samples analyzed represent a mitochondrially enriched proteome rather than a bone fide mitochondrial proteome. However, a total of 288 mitochondrial proteins were identified, of which some 50 showed differential regulation in β°-thalassemia/Hb E erythroblasts as compared to normal control erythroblasts. The proteome data was generated for day 10 of culture cells, representing a time point of significant globin expression as observed in our earlier proteomic analysis [7]. The proteins identified as differentially regulated included those associated with oxidative phosphorylation and energy metabolism, consistent with our earlier observations of dysregulation of these processes in β°-thalassemia/Hb E [7].

Two of the proteins identified as differentially expressed between normal controls and mild and severe β°-thalassemia/Hb E patients were hsp60 and prohibitin 2. Both of these proteins are well defined as mitochondrial proteins, although they also show localization in other cellular compartments, and both proteins are classed as mitochondrial chaperone proteins (reviewed in [15]). Somewhat surprisingly, the proteome data showed discordant expression of these two proteins, with hs60 showing increased expression in severe cases, while prohibitin2 was strongly down regulated in severe cases as compared to both normal controls and mild cases. Studies have shown that both of these proteins are involved in the regulation of apoptosis. Over expression of prohibitin increases cellular tolerance to stimuli that activate mitochondrially mediated apoptosis, while knock down of prohibitin broadly increases sensitivity to apoptosis, although cell type differences have been observed (reviewed in [24]). Hsp60 is believed to function as a pro-apoptotic molecule through its association with caspase-3 [25], although in cardiac myocytes the protein may negatively regulate apoptosis [26]. The main pathophysiology of β°-thalassemia/Hb E is mediated by ineffective erythropoeisis, which is the induction of apoptosis during erythroid differentiation at the polychromatophilic normoblast stage of erythroid differentiation [3]. In this regards the increased expression of the pro-apoptotic protein (hsp60) and the decreased expression of the anti-apoptotic protein (prohibitin2) seen in severe cases of β°-thalassemia/Hb E at day 10 of differentiation would appear to correlate with the main physiopathological process in severe cases of β°-thalassemia/Hb E.

Both confocal microscopy and flow cytometry showed higher levels of mitotracker staining from β°-thalassemia/Hb E erythroblasts as compared to normal control erythroblasts through the period of differentiation examined. However, as shown by confocal microscopy, the pattern of staining was discordant between controls and patients. In particular while day 7 erythroblasts showed essentially perinuclear staining, this was not observed in the thalassemic cells. However, studies have shown that mitotracker has a number of protein targets of which the most significant is hsp60 [20], a protein shown to be differentially regulated in this study, suggesting that quantitation of mitochondria in β°-thalassemia/Hb E should not be undertaken with mitotracker dyes.

Mitochondria are known to be removed during erythroid differentiation by the process of mitophagy and this is believed to occur during reticulocyte maturation [17]. However, our

results with quantitation of relative genome copy number during differentiation show a consistent loss of mitochondria over the entire differentiation period. Interestingly, on day ten of differentiation, the relative numbers of remaining mitochondria in the thalassemic cells were higher than in normal controls, suggesting that mitochondria removal is slightly slower in these cells than in normal controls. This would again possibly be consistent with the increased oxidative phosphorylation see in these cells [7]. Interestingly, three autophagy/mitophagy related proteins (autophagy-related protein 13, E3 ubiquitin-protein ligase RNF 185 and ubiquitin-like modifier-activating enzyme ATG7) were seen as differentially expressed in the mitochondrial proteome, and as autophagy is up-regulated in β°-thalassemia/Hb E erythroblasts as compared to normal control erythroblasts [27], it suggests that selective mitophagy [28], rather than autophagy *per se* is disrupted.

While the relative numbers of mitochondria were higher in day 10 erythroblasts from β°-thalassemia/Hb E patients as compared to normal controls, in terms of activity a different picture emerged. In particular expression of two of the three mitochondrial genes examined was significantly lower on day 10 in the thalassemic cells. Consistent with this, an assessment cellular redox state through the alamarBlue assay showed a significantly decreased signal on day ten in the cells from β°-thalassemia/Hb E patients as compared to normal controls. Interestingly however, examination of newly isolated CD34+ erythroid progenitor cells by the alamarBlue assay showed a markedly higher redox status in both mild and severe β°-thalassemia cells as compared to normal controls, and markedly a significant difference between cells from mild and severe patients.

Conclusion

Combined these results suggest a complex association between mitochondria and the pathology of β°-thalassemia/Hb E as mediated by erythroid differentiation. The results suggest that there is a markedly different redox state in newly isolated CD34+ cells, and this may result from the differing levels of EPO in these patients as compared to normal controls. Effects on mitochondria as seen by mitotracker staining are seen by day 7 of differentiation, and significant deficits in activity are seen on day 10, coincident with significant levels of globin chain synthesis [7]. These results would suggest that the deposition of unpaired globin chains is directly affecting the integrity of mitochondria. As there are more mitochondria present in cells from thalassemia patients on day 10, it suggests that the effect is magnified, with the damage to mitochondria at this point in time being co-incident with the onset of apoptosis in ineffective erythropoiesis [3].

Supporting Information

S1 File. Fig A. Western blot of NDUFA9 showing enrichment of mitochondrial proteins. Fig B. SDS-PAGE of mitochondria enriched proteins. Fig C. Hierarchical clustering analysis of 288 mitochondial proteins. Fig D. Original unmerged and merged images from Fig 3. Fig E. Original scatterplots of flow cytometry as presented in Fig 3. Table A. Specific primer sequences and cycle conditions. Table B. Significantly differentially expressed mitochondrial proteins in β°-thalassemia/Hb E erythroblasts.
(PDF)

Author Contributions

Conceived and designed the experiments: KK WS DRS. Performed the experiments: KK WS AP JJ. Analyzed the data: KK WS PL SR DRS. Contributed reagents/materials/analysis tools: SS SF. Wrote the paper: KK DRS.

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REVIEW

Analysis of protein profiling studies of β-thalassemia/Hb E disease

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A number of studies have used global protein profiling technologies on a range of patient samples to detect proteins that are differentially expressed in β-thalassemia/Hb E as an aid for understanding the physiopathology of this disease. Seven studies have identified a total of 111 unique, differentially expressed proteins. Seven proteins (prothrombin, alpha-1-antichymotrypsin, fibrinogen beta chain, hemoglobin beta, selenium-binding protein, microtubule-actin crosslinking factor and adenomatous polyposis coli protein 2) have been identified in two independent studies, whereas two proteins (carbonic anhydrase 1 and peroxiredoxin-2) have been identified in three independent studies. Both of these latter two proteins were consistently upregulated in the studies that identified them. Ontological analysis of all differentially regulated proteins identified "response to inorganic substances" as the most significant functional annotation cluster, which is consistent with iron overload being a major pathological consequence of this disease. Despite the range of samples investigated and the relatively small number of studies undertaken, a coherent picture of the mediators of the pathological consequences of β-thalassemia/Hb E disease is starting to emerge.

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1 Introduction

The thalassemias are a group of hereditary blood disorders associated with the defective production of hemoglobin, and are among the most prevalent hemoglobinopathies worldwide with a distribution encompassing the Mediterranean region, the Indian subcontinent, Southeast Asia and West Africa [1]. The prevalence of this genetic disorder varies among populations and ethnic groups depending on the type of thalassemia investigated. Although there are several forms of thalassemia, the two most common forms (alpha (α) and beta (β)-thalassemia) have been identified as public health concerns in several regions, especially in developing countries including Thailand where approximately 30% of the population has been reported to carry aberrant α- or β-globin genes leading to significant financial loss due to morbidity and mortality [2, 3].

Although the under production of hemoglobin as a consequence of defective globin chains and the inheritance pattern (autosomal recessive) observed in both α- and β-thalassemia are essentially the same, there are distinct features that clearly differentiate one from another. First, in α-thalassemia, functional loss frequently arises from gross gene deletions of the α-globin genes located on chromosome 16 (each allele contains two α-globin genes), whereas β-thalassemia is characterized by base substitutions in the single copy β-globin gene located on chromosome 11 [4, 5]. Second, numerous studies have indicated that the worldwide distribution of α- and β-thalassemia is strongly correlated with areas with past and/or present malaria endemicity, as a consequence of natural selection leading to a high prevalence in tropical and subtropical areas, and it is well documented that homozygous α-thalassemia confers significant protection against severe malaria [6]. While data on β-thalassemia are scarce, one study reported no significant correlation between β-thalassemia trait and the incidence of malaria in Liberia [7]. Finally, the molecular pathophysiology of thalassemia results from the imbalance between the α- and β-globin chains. α-thalassemia

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Abbreviations: **IMAC**, immobilized metal affinity chromatography; **MALDI-MS**, matrix assisted laser desorption ionisation mass spectrometry; **SDS**, Sodium dodecyl sulfate

Colour Online: See the article online to view Fig. 1 in colour.

is associated with a relative excess of β-globin chains, which form soluble β_4 tetramers (Hb H) that accumulate inside red cells leading to increased hemolysis, while in contrast the insoluble excess α-globin chains (resulting from the reduced or absent production of β-globin chains) are highly unstable and toxic. As a consequence, the premature destruction of differentiating erythroblasts occurs in a process known as ineffective erythropoeisis [8], in addition to increased hemolysis of the small number of red blood cells that reach maturity. It should be noted that the lower cellular toxicity of the β_4 tetramer (α-thalassemia) as compared to unbound α-globin chains (β-thalassemia) might underpin why the clinical manifestation of β-thalassemia is usually more severe than that of an α-thalassemia of comparable genetic severity, except for the complete loss of all 4 alpha globin gene copies (homozygous α^0-thalassemia) which is the most severe form of thalassemia (Hb Bart's hydrops fetalis syndrome) from which affected infants usually die *in utero* [5, 8, 9]. The life-threatening chronic anemia frequently observed in β-thalassemia patients requires regular blood transfusions, and the consequent iron overload is a major cause of morbidity and mortality of β-thalassemia patients [10, 11].

2 β-thalassemia/Hb E disease

β-thalassemia refers to a group of hereditary blood disorders that are characterized by either reduced (β^+) or absent (β^0) production of β-globin chains. Approximately, 200 disease causing mutations associated with the β-globin gene have been reported [4, 12]. The clinical severity of β-thalassemia can be simply classified into three categories, including β-thalassemia minor, intermediate and major, however, the prediction of clinical phenotype from a known genotype of an individual with β-thalassemia is not straightforward, especially in the inherited compound heterozygous disease β-thalassemia/Hb E [4, 12, 13].

β-thalassemia/Hb E is highly prevalent in many parts of Southeast Asia particularly in Thailand, Laos and Cambodia, where the allele frequency of either hemoglobin E or β-thalassemia is unusually high. The co-inheritance of those two alleles is therefore common in the populations residing in such areas [14]. The disease is associated with a wide range of presentation, ranging from a mild disease with slight anemia to a severe, transfusion-dependent anemia [15]. Even after accounting for known compensating genetic factors such as co-inheritence of α-globinopathies, there remains a spectrum of presentation that cannot be fully accounted for [16, 17].

Importantly, β-thalassemia/Hb E represents the largest (30–50%) class of thalassemia patients whose symptoms are associated with severe clinical phenotypes [18, 19]. The pathophysiology of β-thalassemia/Hb E is primarily involved with the reduced β-globin chain synthesis caused by the inheritance of a β-thalassemia allele (β^+ or β^0) from one parent with the β^E allele derived from the other. The β^E structural globin variant arises from the substitution of a lysine for a glutamic acid at codon 26 of β-globin, resulting in the structurally abnormal β^E globin chain which is unstable. The compound heterozygote (β-thalassemia/Hb E) physiologically gives rise to an imbalanced α and β-globin chain ratio, resulting in ineffective erythropoiesis, oxidative damage and shortened survival of red blood cells [13].

Although ineffective erythropoiesis is considered as the major hallmark of β-thalassemia, iron overload is the primary cause of mortality in transfusion-dependent β-thalassemia [10, 11]. However, iron overload is also common in transfusion-independent β-thalassemia and this results in several complications, including hepatic and endocrine disorders [20]. Studies have shown hepcidin, the critical iron regulating peptide produced in the liver is a key player mediating iron overload in β-thalassemia and β-thalassemia/Hb E diseases [21, 22]. Hepcidin functions by binding to the sole iron exporting protein, ferroportin, and promoting its internalization and degradation by lysosomes [23]. Under high levels of hepcidin expression, iron is therefore held within iron storage cells, while under low levels of hepcidin iron is exported from these cells to be available for erythropoiesis [23]. However, low levels of hepcidin [22], as well as dysregulation of ferroportin expression by erythroid precursor cells have been reported in β-thalassemia/Hb E patients [24], suggesting significant defects in iron homeostasis mechanisms in these patients.

Given the complexity of the molecular pathophysiology associated with β-thalassemia/Hb E disease, proteomic analysis has increasingly been applied for understanding the pathophysiology of β-thalassemia/ Hb E disease. However, the diversity of these studies with regards to both their methodology and starting material make interpretation of broad consensus difficult, and this review aims to provide an overview so that candidate proteins of significant interest do not get missed in the experimental noise generated by these studies. In particular our approach differs from previous reviews that have tended to focus on particular sub-sets of studies such as red cell proteins [25] and erythrocytes and platelets [26] in the broader context of hemoglobinopathies.

3 Studies

A total of seven studies [27–33] have investigated differential protein expression in a range of patient specimens (Table 1). Most of the studies have investigated differences between normal controls and β-thalassemia/Hb E patients [27–29, 31, 33], while in one study four cohorts (Normal control, β-thalassemia/Hb E non-splenectomized, β-thalassemia/Hb E splenectomized and β-thalassemia) were investigated [30]. Hatairaktham et al. additionally compared between mild and severe forms of β-thalassemia/Hb E [29]. The studies variously analyzed platelets [30], erythrocytes [27], platelet-free plasma derived microparticles [28], plasma [33], erythroid progenitor cells [31] and bone marrow derived CD34+ cells [32]. The predominant separation methodology employed

Table 1. Summary of proteomic studies of β-thalassemia/Hb E disease

Study [reference]	Year published	Material	Method	# of different proteins identified as differentially regulated
Differential protein expression				
Bhattacharya et al. [20]	2010	Erythrocytes	2-DE and MALDI-MS/MS	13 cytosolic 7 membrane
Leecharoenkiat et al. [24]	2011	Peripheral blood derived erythroid precursor cells	2-DE and LC-MS/MS	18[a]
Chaichompoo et al. [21]	2012	Platelet free plasma derived microparticles	2-DE and Q-TOF MS and MS/MS	9 reduced expression 11 increased expression 7 only in β-thalassemia[b]
Weeraphan et al. [26]	2013	Plasma	2D-DIGE and LC-MS/MS	6 proteins 1 with altered carbonyl content
Hatairaktham et al. [22]	2013	Plasma	2-DE and MALDI-MS/MS	31 unique proteins
Karmakar et al. [23]	2015	Platelets	2-DE and 2D-DIGE and MS and MS/MS	5 proteins
Differential phosphoproteins				
Ponnikorn et al. [25]	2011	Bone marrow derived erythroid precursor cells	IMAC phosphoprotein isolation and LC-MS/MS	266 proteins

[a] 17 proteins were identified as differentially expressed during erythropoiesis, 18 proteins as differentially expressed in comparing between β-thalassemia/Hb E and normal controls.
[b] One protein (hemoglobin mu chain was identified as both increased and present only in β-thalassemia).

was two-dimensional gel electrophoresis (2-DE; [34]) which was used by five studies [27–31], although one study [30] additionally employed two-dimensional difference in gel electrophoresis (2D-DIGE), a technique solely employed by Weeraphan et al. [33]. One study utilized immobilized metal affinity chromatography (IMAC) to select phosphoproteins with no further protein separation [32].

2-DE is undertaken by first separating proteins in a lysate by their isoelectric point, and subsequently further separating the proteins by electrophoresis through an SDS-polyacrylamide gel [34]. The dual separation can result in the resolution of many hundreds of proteins, which can be identified through subsequent mass spectroscopic analysis. 2-DE DIGE is a refinement of the 2-DE methodology, which allows for improved sensitivity to detect less abundant proteins [35, 36]. In 2-DE, proteins from different sources are separated in parallel, while in 2-DE DIGE proteins from different sources are labeled with different, charge matched, fluorescent dyes before pooling and separation by 2-DE [37]. The sensitivity increase over standard 2-DE has been estimated to be on the order of 10- to 40-fold [37]. Both 2-DE and 2-DE DIGE utilize image analysis to determine spots differentially expressed between the samples.

Identification of the differently regulated proteins was undertaken by a variety of mass spectroscopy methods, including matrix-assisted laser desorption ionization (MALDI)-tandem mass spectroscopy (MALDI-MS/MS) [27, 29], nano-scale liquid chromatography-tandem mass spectroscopy (LC-MS/MS) [31–33], quadrapole-time of flight mass spectroscopy (Q-TOF MS) and tandem mass spectroscopy (MS/MS) [28] and mass spectroscopy and tandem mass spectroscopy (MS and MS/MS) [30].

The earliest study by Bhattacharya et al. was undertaken on erythrocytes from 14 β-thalassemia/Hb E patients and six normal controls, and the authors used 2-DE and MALDI-MS/MS to identify differentially expressed proteins [27]. The authors investigated both complete erythrocytes after Hb depletion as well as erythrocyte ghosts to provide a clearer

analysis of membrane bound proteins, and a total of 13 cytosolic proteins and seven membrane bound proteins were identified as differentially expressed between normal control erythrocytes and erythrocytes from β-thalassemia/Hb E patients [27].

Leecharoenkiat et al. investigated differentially expressed proteins between erythroid precursor cells from β-thalassemia/Hb E patients and normal controls using a combination of 2-DE and nano-LC-MS/MS [31]. Cells were generated by the isolation of CD34+ hematopietic stem cells from peripheral blood, and culture under a protocol driving erythropoiesis. The authors identified 17 proteins differentially expressed during erythropoiesis as well as 18 proteins that were differentially expressed between erythroid precursor cells from normal controls and β-thalassemia/Hb E patients [31]. As noted by the authors, the majority of differentially expressed proteins function in glycolysis and the tricarboxylic acid cycle (TCA) cycle. In this review, only proteins identified as differentially expressed between normal and β-thalassemia/Hb E patients were included in analyses.

Combined, the studies of Bhattacharya et al. [27] and Leecharoenkiat et al. [31] identified a total of 33 unique proteins, of which 4 (carbonic anhydrase, peroxiredoxin-2, β-globin and selenium-binding protein) were identified by both studies. Both studies showed carbonic anhydrase and peroxiredoxin-2 were increased in expression in β-thalassemia/Hb E as compared to normal controls, while discordant results were seen for both selenium-binding protein and β-globin (Table 2). The discordant result for β-globin was surprising, as Leecharoenkiat et al. showed β-globin expression to be reduced as compared to normal controls [31], while Bhattacharya et al. reported increased expression [27]. However, Bhattacharya et al. identified β-globin as increased in erythrocyte ghosts, and it is possible that the β-globin E chain is stickier on membranes than the normal β-globin protein [8], leading to its increased presence. Additionally however, Bhattacharya et al. employed Hb depletion as part of their protein purification protocol, which would serve to complicate the subsequent analysis [27].

Chaichompoo et al. analyzed the expression of proteins in platelet-free plasma derived microparticles derived from 15 splenectomized β-thalassemia/Hb E patients and 15 age matched healthy controls using 2-DE coupled with Q-TOF MS and MS/MS [28]. The authors identified 9 proteins with reduced expression, 11 proteins with increased expression and 7 proteins that were identified only in β-thalassemia/Hb E derived microparticles, however somewhat confusingly, hemoglobin mu chain is listed both as a protein increased in expression, and as a protein found only in β-thalasemia/Hb E derived microparticles. In total therefore the authors identified 26 proteins as differentially expressed in microparticles derived from β-thalassemia/Hb E patients as compared to those derived from normal controls [28]. One protein, carbonic anhydrase I, identified in this study as increased in expression microparticles from β-thalassemia/Hb E as compared to normal controls had been similarly identified as increased in β-thalassemia/Hb E as compared to normal controls by the two earlier studies [27, 31] (Table 2).

A more recent article has investigated erythrocyte released microparticles [38], and as part of the study the authors characterized the microparticle associated proteins using gel-enhanced nano-scale liquid chromatography with tandem mass spectroscopy (GelC-MS/MS [39]). However, as the analysis investigated erythrocyte released microparticles from thalassemia patients of mixed genotypes, the results were not included in this analysis. It is worth noting however that carbonic anhydrase was one of the proteins detected in microparticles released from thalassemic erythrocytes as well as in microparticles from phenylhydrazine-treated normal control erythrocytes [38].

Weeraphan et al. investigated the effects of curcuminoids in reducing oxidative modifications of the plasma proteome in β-thalassemia/Hb E patients, and as part of the study determined the baseline plasma proteomes from normal controls and β-thalassemia/Hb E patients [33]. Using 2-DE DIGE and LC-MS/MS the authors identified 26 differentially expressed protein spots which resolved to 6 different proteins (prothrombin, 5 isoforms of hemopexin, 4 isoforms each of fibrinogen α- and β-chains, 2 isoforms of fibrinogen γ-chain, 7 isoforms of haptoglobin β-chain and 3 isoforms of haptoglobin α-chain) [33]. Authors also identified α-1-antichymotrypsin as a protein with altered carbonyl content. One of the proteins identified by these authors as expressed at lower levels in β-thalassemia/Hb E patients as compared to normal controls, fibrinogen β-chain, had also been identified by Chaichompoo et al. as expressed less in microparticles from β-thalassemia/Hb E patients as compared to those from controls [28] (Table 2).

Hatairaktham et al. also investigated the plasma proteome in β-thalassemia/Hb E patients, and in addition to comparing between normal control and patients, the authors investigated differences between mild and severe presentation of β-thalassemia/Hb E [29]. Using a combination of 2-DE and MALDI-MS/MS the authors identified 29 protein spots as differently expressed between patients and normal controls, and an additional 9 spots as differentially expressed between mild and severe β-thalassemia/Hb E. These resolved to 24 and 9 unique proteins, respectively, after removing proteins multiply identified, although we were unable to identify one protein (spot number 618 "hypothetical protein") as the NCBI ID number given in the paper has been formally removed from the NCBI database, and blasting the sequence did not result in a definitive protein identification in UniProt database [40, 41]. One protein, α-1-antichymotrypsin was identified twice, once as down regulated in patients in comparison to controls, and once in comparison between mild and severe β-thalassemia patients, and therefore a total of 31 different proteins were identified in the study. Three proteins identified in this study, namely prothrombin, α-1-antichymotrypsin and microtubule-actin cross-linking factor 1 had been identified in earlier studies, two (prothrombin and α-1-antichymotrypsin) in the study

Table 2. Summary of proteins identified by more than one proteomic analysis of β-thalassemia/ Hb E

	Protein	UniProt	Studies [reference]
Proteins identified in two studies			
1	prothrombin	P00734	Hatairaktham et al. [22] Weeraphan et al. [26]
2	alpha-1-antichymotrypsin	P01011	Hatairaktham et al. [22] Weeraphan et al. [26]
3	fibrinogen beta chain	P02675	Chaichompoo et al. [21] Weeraphan et al. [26]
4	hemoglobin beta	P68871	Bhattacharya et al. [20] Leecharoenkiat et al. [24]
5	selenium-binding protein	Q13228	Bhattacharya et al. [20] Leecharoenkiat et al. [24]
6	microtubule-actin cross linking factor	Q9UPN3	Chaichompoo et al. [21] Hatairaktham et al. [22]
7	adenomatous polyposis coli protein 2	O95996	Hatairaktham et al. [22] Ponnikorn et al. [25]
Proteins identified in three studies			
1	carbonic anyhdrase 1	P00915	Bhattacharya et al. [20] Chaichompoo et al. [21] Leecharoenkiat et al. [24]
2	peroxiredoxin-2	P32119	Bhattacharya et al. [20] Karmakar et al. [23] Leecharoenkiat et al. [24]

of the plasma proteome by Weeraphan et al. [33], and the remaining protein by Chaichompoo and colleagues in the study undertaken on microparticles [28] (Table 2).

A recent study undertaken by Karmakar et al. investigated differences in protein expression in the platelet proteome from splenectomized and non-splenectomized β-thalassemia/Hb E patients and β-thalassemia patients all compared to normal controls using a combination of 2-DE and 2-DE DIGE together with MS and MS/MS [30]. The authors identified five proteins as commonly increased in expression in the thalassemia patients, namely Hsp70, protein disulfide-isomerase, eukaryotic translation initiation factor 5A-1, peroxiredoxin-2 and superoxide dismutase [Cu-Zn]. Of these proteins, only peroxiredoxin-2 had been previously identified [29, 31], and in all cases peroxiredoxin-2 was observed to be increased in expression in β-thalassemia/Hb E specimens as compared to specimens from normal controls [29–31] (Table 2).

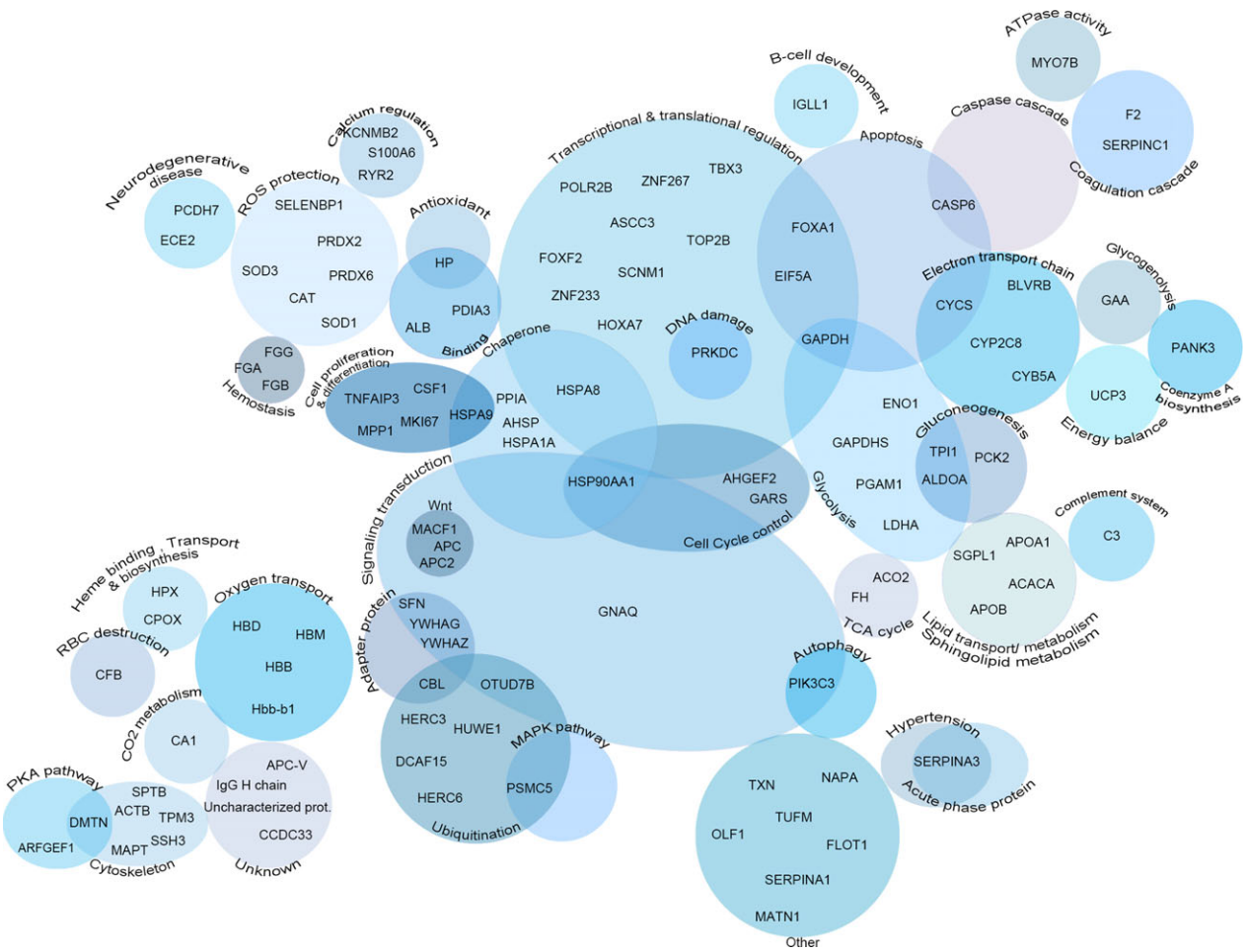
The bone marrow is the major site of erythropoiesis [42], and while to date no un-enriched proteomic analysis has been undertaken on differences in protein expression levels in cells taken from bone marrow of β-thalassemia/Hb E patients and normal controls, Ponnikorn et al. have undertaken a proteomic analysis of phosphoprotein enriched proteins [32]. The authors selected CD34+ cells from bone marrow samples of three β-thalassemia/Hb E patients and two normal controls and enriched for phosphoproteins through immobilized metal affinity chromatography (IMAC) and analyzed the resulting proteins by LC-MS/MS. A total of 266 proteins were identified, from 347 peptides of which roughly 60% were identified by bioinformatic analysis as phosphopeptides. While the authors provided a full list of all 226 proteins, only selected expression data for some proteins (determined from peptide intensities) was provided. From the data provided, 17 proteins were identified as differentially expressed (set by the authors as an average peptide abundance ratio of $>$ 2-fold) in β-thalassemia/Hb E as compared to normal controls. One of these proteins, adenomatous polyposis coli-2 (APC2) had been previously identified by Hatairaktham et al. [29], but while that study identified APC2 as decreased in expression in β-thalassemia/Hb E as compared to normal controls, the study by Ponnikorn et al. showed APC to be increased in expression in β-thalassemia/Hb E as compared to normal controls [32] (Table 2).

Between the seven studies [27–33], a total of 111 unique proteins that were differentially expressed between specimens from normal controls and β-thalassemia/Hb E patients were identified. Ontological analysis using the on-line DAVID bioinformatics resource [43, 44] identified "response to inorganic substances" as the most significant process (P value of 1.6×10^{-11}) in annotation cluster 1, which is consistent with the known pathophysiology of β-thalassemia/Hb E in which iron overload is the most significant pathological consequence of β-thalassemia/Hb E [45]. All together the DAVID resource identified 61 annotation clusters, with the most significant processes of "acute phase" (P value of 1.3×10^{-10})

and "membrane-bound vesicles" (P value of 1.2×10^{-6}) being found in annotation clusters 2 and 3, respectively. A further mapping of the proteins using the UniProtKB [41] mapping function identified a number of processes, including transcriptional and translational regulation, apoptosis, signal transduction and electron transport chain functions (see Fig. 1). While very few proteins were commonly identified by the different studies, there is a more robust grouping of proteins by functional mapping as seen in Fig. 1. This again supports a previous proposal that studies that investigate differentially expressed proteins are likely to identify different proteins but may highlight common biological processes [46].

Only two proteins were identified by more than two studies, with both peroxiredoxin-2 and carbonic anhydrase I having been identified as increased in expression in β-thalassemia/Hb E specimens as compared to specimens from normal controls by three studies each. Both of these proteins were also shown to be highly upregulated in a study of erythroid precursor cells of homozygous β^{0cod39} patients as compared to normal controls [47], suggesting that elevation of expression of these two proteins may be a common event in β-thalassemia. Neither of these two proteins were shown to be increased in expression in studies undertaken on erythroid progenitor cells [48] and plasma [49] from the α-hemoglobinopathy, Hemoglobin H-Constant Spring (Hb H-CS) as compared to normal control samples.

Peroxiredoxin-2 has been shown to be increased in expression in erythroid precursor cells [31], erythrocytes [27] and platelets [30] derived from β-thalassemia/Hb E patients as compared to those derived from normal controls. Platelets derive from the same common myeloid progenitor cell (which arise from multipotent hematopoietic stem cells) as erythrocytes, but platelets are generated by differentiation of these cells into megakaryoblasts, promegakaryocytes and finally megakaryocytes, while cells in the erythroid lineage differentiate into proerythroblasts before differentiating and maturing to erythrocytes [50], and thus increased expression of peroxiredoxin-2 in β-thalassemia may be lineage associated. Peroxiredoxin-2 is a 2-cystein member of the peroxiredoxin family of antioxidant enzymes, and peroxiredoxin-2 is cytoprotective as it is able to efficiently scavenge H_2O_2 at low concentrations [51]. Several studies have shown increased reactive oxygen species during erythropoiesis in β-thalassemia/Hb E [31,52], and it has been



proposed that the increased expression of peroxiredoxin-2 is a cytoprotective mechanism in β-thalassemia [47], possibly working through an interplay with nuclear factor-erythroid 2 [53].

Carbonic anhydrase I mediates the reversible hydration of carbon dioxide to bicarbonate and hydrogen ions [54]. It has been shown to be increased in expression in erythroid precursor cells [31], erythrocytes [27] and in microparticles [28] derived from β-thalassemia/Hb E patients as compared to those derived from normal controls. While there are several different carbonic anhydrases, in mature red blood cells, carbonic anhydrase I is the major component. Early studies suggested a possible link between carbonic anhydrase I deficiency and increased levels of HbF in some disorders [55, 56], but while later studies confirmed that carbonic anhydrase was increased in thalassemia, no direct evidence of a link with HbF levels was shown [57]. Interestingly, carbonic anhydrase expression is closely linked with the switch from fetal to adult hemoglobin production in erythroid cells [58], and this is probably regulated through the Ldb1 protein complex [59] the primary mediator of global erythroid gene activation [60, 61]. The increased expression of carbonic anhydrase I in cells with decreased hemoglobin expression possibly indicates a mechanism by which hemoglobin levels are detected in the cells to control levels of carbonic anhydrase I.

Very recently, one further partial β-thalassemia/Hb E proteome has been published. In this study, Khungwanmaythawee et al. investigated the differential expression of mitochondrial proteins isolated from normal controls and β-thalassemia/Hb E patients of different severities [62]. Although 50 differentially regulated mitochondrial proteins were identified, none of them matched any protein identified by the earlier studies.

4 Conclusions

While only a relatively small number of studies have investigated β-thalassemia/Hb E disease using proteomic approaches, they have all investigated materials derived from patients. Despite the wide range of samples investigated, there is some consensus in that "response to inorganic substances" was the most significant gene ontology cluster when all identified differentially regulated proteins were included in the analysis, and this agrees well with the known impact of iron overloading in both transfusion-dependent and transfusion-independent β-thalassemia/Hb E patients. Both of the proteins identified by three independent studies are deserving of greater investigation to understand the

◀ **Figure 1.** Diagrammatic representation of ontological analysis of proteins identified as differentially regulated in proteomic analysis studies of β-thalassemia/Hb E disease. Abbreviations in the figure: ACACA (Acetyl-CoA carboxylase 1), ACO2 (Aconitate hydratase, mitochondrial), ACTB (Actin, cytoplasmic 1), AHSP (Alpha-hemoglobin-stabilizing protein), ALB (Serum albumin), ALDOA (Fructose-bisphosphate aldolase A), APC (Adenomatous polyposis coli protein), APC-V (APC variant protein), APC2 (Adenomatous polyposis coli protein 2), APOA1 (Apolipoprotein A-I), APOB (Apolipoprotein B-100), ARFGEF1 (Brefeldin A-inhibited guanine nucleotide-exchange protein 1), ARHGEF2 (Rho guanine nucleotide exchange factor 2), ASCC3 (Activating signal cointegrator 1 complex subunit 3), BLVRB (Flavin reductase (NADPH)), C3 (Complement C3), CA1 (Carbonic anhydrase 1), CASP6 (Caspase-6), CAT (Catalase), CBL (E3 ubiquitin-protein ligase CBL), CCDC33 (Coiled-coil domain-containing protein 33), CFB (Complement factor B), CPOX (Oxygen-dependent coproporphyrinogen-III oxidase, mitochondrial), CSF1 (Macrophage colony-stimulating factor 1), CYB5A (Cytochrome b5), CYCS (Cytochrome c), CYP2C8 (Cytochrome P450 2C8), DCAF15 (DDB1- and CUL4-associated factor 15), DMTN (Dematin), ECE2 (Endothelin-converting enzyme 2), EIF5A (Eukaryotic translation initiation factor 5A-1), ENO1 (Alpha-enolase), F2 (Prothrombin), FGA (Fibrinogen alpha chain), FGB (Fibrinogen beta chain), FGG (Fibrinogen gamma chain), FH (Fumarate hydratase, mitochondrial), FLOT1 (Flotillin-1), FOXA1 (Hepatocyte nuclear factor 3-alpha), FOXF2 (Forkhead box protein F2), GAA (Lysosomal alpha-glucosidase), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), GAPDHS (Glyceraldehyde-3-phosphate dehydrogenase, testis-specific), GARS (Glycine–tRNA ligase), GNAQ (Guanine nucleotide-binding protein G(q) subunit alpha), HBB (Hemoglobin subunit beta), Hbb-b1 (Hemoglobin subunit beta-1), HBD (Hemoglobin subunit delta), HBM (Hemoglobin subunit mu), HERC3 (Probable E3 ubiquitin-protein ligase HERC3), HERC6 (Probable E3 ubiquitin-protein ligase HERC6), HOXA7 (Homeobox protein Hox-A7), HP (Haptoglobin), HPX (Hemopexin), HSP90AA1 (Heat shock protein HSP 90-alpha), HSPA1A (Heat shock 70 kDa protein 1A), HSPA8 (Heat shock cognate 71 kDa protein), HSPA9 (Stress-70 protein, mitochondrial), HUWE1 (E3 ubiquitin-protein ligase HUWE1), IgG H chain (Immunoglobulin G heavy chain), IGLL1 (Immunoglobulin lambda-like polypeptide 1), KCNMB2 (Calcium-activated potassium channel subunit beta-2), LDHA (L-lactate dehydrogenase A chain), MACF1 (Microtubule-actin cross-linking factor 1), MAPT (Microtubule-associated protein tau), MATN1 (Cartilage matrix protein), MKI67 (Antigen KI-67), MPP1 (55 kDa erythrocyte membrane protein), MYO7B (Unconventional myosin-VIIb), NAPA (Alpha-soluble NSF attachment protein), OLF1 (Olfactory receptor-like protein), OTUD7B (OTU domain-containing protein 7B), PANK3 (Pantothenate kinase 3), PCDH7 (Protocadherin-7), PCK2 (Phosphoenolpyruvate carboxykinase [GTP], mitochondrial), PDIA3 (Protein disulfide-isomerase A3), PGAM1 (Phosphoglycerate mutase 1), PIK3C3 (Phosphatidylinositol 3-kinase catalytic subunit type 3), POLR2B (DNA-directed RNA polymerase II subunit RPB2), PPIA (Peptidyl-prolyl cis-trans isomerase A), PRDX2 (Peroxiredoxin-2), PRDX6 (Peroxiredoxin-6), PRKDC (DNA-dependent protein kinase catalytic subunit), PSMC5 (26S protease regulatory subunit 8), RYR2 (Ryanodine receptor 2), S100A6 (Protein S100-A6), SCNM1 (Sodium channel modifier 1), SELENBP1 (Selenium-binding protein 1), SERPINA1 (Alpha-1-antitrypsin), SERPINA3 (Alpha-1-antichymotrypsin), SERPINC1 (Antithrombin-III), SFN (14-3-3 protein sigma), SGPL1 (Sphingosine-1-phosphate lyase 1), SOD1 (Superoxide dismutase [Cu–Zn]), SOD3(Extracellular superoxide dismutase [Cu–Zn]), SPTB (Spectrin, beta, erythrocytic), SSH3 (Protein phosphatase Slingshot homolog 3), TBX3 (T-box transcription factor TBX3), TNFAIP3 (Truncated tumor necrosis factor alpha-induced protein 3), TOP2B (DNA topoisomerase 2-beta), TPI1 (Triosephosphate isomerase), TPM3 (Tropomyosin alpha-3 chain), TUFM (Elongation factor Tu, mitochondrial), TXN (Thioredoxin), UCP3 (Mitochondrial uncoupling protein 3), YWHAG (14-3-3 protein gamma), YWHAZ (14-3-3 protein zeta/delta), ZNF233 (Zinc finger protein 233), ZNF267 (Zinc finger protein 267).

significance of over-expression, and how this is a consequence of the pathology of the disease. While the relationship between increased reactive oxygen species [31, 52] and increased expression of peroxiredoxin-2 is perhaps fairly straightforward, the increased expression of carbonic anhydrase I might have less clear implications, particularly as this protein comes under regulatory control of the global erythroid gene activation complex.

It is well known that β-thalassemia/Hb E can be cured by allogenic bone marrow transplant [63], and efforts are being made to develop curative treatments with novel genome manipulation technologies [64]. However, a number of β-thalassemia/Hb E patients have the potential to have their quality of life improved through a relatively modest decrease in the severity of anemia [65]. In this regards proteomic analysis is helping to determine targets amenable for intervention that can alleviate the degree ineffective erythropoiesis, easing the severity of anemia. In addition, better alleviation of iron overloading will be of benefit to both transfusion-dependent and transfusion-independent β-thalassemia/ Hb E patients.

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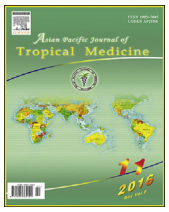

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Iron dysregulation in beta-thalassemia

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ABSTRACT

Iron deficiency anemia and iron overload conditions affect more than one billion people worldwide. Iron homeostasis involves the regulation of cells that export iron into the plasma and cells that utilize or store iron. The cellular iron balance in humans is primarily mediated by the hepcidin–ferroportin axis. Ferroportin is the sole cellular iron export protein, and its expression is regulated transcriptionally, post-transcriptionally and post-translationally. Hepcidin, a hormone produced by liver cells, post-translationally regulates ferroportin expression on iron exporting cells by binding with ferroportin and promoting its internalization by endocytosis and subsequent degradation by lysosomes. Dysregulation of iron homeostasis leading to iron deposition in vital organs is the main cause of death in beta-thalassemia patients. Beta-thalassemia patients show marked hepcidin suppression, ineffective erythropoiesis, anemia and iron overload. Beta-thalassemia is common in the Mediterranean region, Southeast Asia and the Indian subcontinent, and the focus of this review is to provide an update on the factors mediating hepcidin related iron dysregulation in beta-thalassemia disease. Understanding this process may pave the way for new treatments to ameliorate iron overloading and improve the long term prognosis of these patients.

1. Introduction

With the exception of a few species of bacteria, all living things need iron as an absolute requirement for viability. The ability of iron to act as both an electron donor and an electron acceptor makes it a critical component of many cellular oxidation/reduction reactions, and in addition iron is the substrate for heme, the critical component of hemoglobin, the essential oxygen carrying molecule of all vertebrates [1]. However, free iron is potentially extremely toxic to cells. Iron can donate electrons to oxygen resulting in the formation of the reactive superoxide radical (O^{2-}) or to hydrogen peroxide generating the hydroxyl (•OH) radical [1], and these molecules can oxidize biological macromolecules including lipids, proteins and DNA with extremely damaging consequences to the cell [2].

Humans contain approximately 3–4 g of iron in various forms [3]. Although iron is extremely plentiful in the environment, much of it is present in insoluble, non-bioavailable forms, and so humans have evolved to be highly efficient in conserving iron. Indeed, humans have no mechanism for excretion of excess iron under conditions of iron overload. Bioavailable iron in the diet serves mainly to replace iron lost from the body through processes such as the shedding of cells from the surface of the skin and lumen of the gut as part of the normal process of epithelial cell turnover. Additional loss of iron

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from the body may occur through minor bleeding events. In general it is believed that only some.

1–2 mg of iron (less than 0.1% of the total iron in a body) are lost from the body each day that require replacement though dietary sources [3].

The majority of the iron in humans is in the form of hemoglobin in red blood cells, and red blood cells combined contain between 2 g and 2.5 g of iron (out of 3–4 g in total). Red blood cells have a life span of some 120 days under normal conditions [2], after which they are degraded by macrophages and the iron returned to the plasma. Plasma contains some 2–3 mg of iron bound to a protein called transferrin, which is the primary molecule that transports iron for use in erythropoiesis in the bone marrow and by other iron requiring cells. Macrophages return some 20–25 mg of iron daily, ensuring a rapid turnover of iron in the plasma. When the binding capacity of serum transferrin is exceeded, iron starts to make complexes with other plasma proteins and molecules such as citrate. This iron is generally termed non-transferrin bound iron (NTBI) [4]. NTBI is easily taken up by hepatocytes and other parenchymal cells, and the intracellular accumulation of iron in these cells rapidly causes damage through oxidation reactions [2].

Within cells, iron is normally stored as the ferric (Fe^{3+} form) in association with a globular protein complex called ferritin. Ferritin is essentially a hollow sphere in which can sequester up to 4500 iron atoms. The ferritin complex consists of 24 subunits of heavy (H) and light (L) chains the exact composition of which can vary between tissues. The H chain has ferroxidase activity which converts Fe^{2+} to Fe^{3+} for storage inside the shell, while the L chain primarily stabilizes the structure and facilitates transport of iron ions to the inside of the structure [1]. This formation is the main storage system of iron (outside of the iron in hemoglobin). Under conditions of iron deficiency, iron is released from the complex to the plasma, while under conditions of mild iron excess the system can provide some buffering against the increased iron levels. In the average male, one gram of iron is held in storage mostly in hepatocytes and macrophages in the liver but also in spleen red pulp macrophages. Women of reproductive age tend to have significantly lower stored iron as a consequence of menstruation and childbearing [5].

As noted above, only a very small fraction of the total iron content is lost daily, and this is replaced through bioavailable iron sourced from the diet. Iron from the diet can be obtained from heme based sources (found in meat) and from non-heme iron sources (iron in cereals, vegetables, pulses etc). Iron absorption takes place in the gut duodenum and upper jejunum and occurs by transport across the apical membrane of enterocytes, which appears to occur through two independent pathways [6], one for heme iron and one for non-heme iron [3]. While absorption of non-heme iron is fairly well understood, the absorption of heme iron and ferritin iron is rather less well understood. Dietary non-heme iron is normally in the form of ferric iron (Fe^{3+}) which is reduced in the gut to the ferrous (Fe^{2+}) form by ferric reductase activity provided by duodenal cytochrome B and possibly Steap2 [3]. The ferrous iron is then transported across the apical (gut lumen) side of enterocytes by the ferrous iron transporter divalent metal ion transporter 1 (DMT-1), also known as Nramp-2 (natural resistance-associated macrophage protein) [7]. Some evidence suggests that heme iron may be taken up by receptor mediated endocytosis, although no high-affinity heme receptor has been identified to date [3]. There is some evidence that dietary ferritin is also taken up by endocytosis [8]. Once inside the enterocyte heme is broken down by heme oxygenase and dietary ferritin iron is released from ferritin. It is currently believed that iron from the various sources enters a common iron pool within the enterocyte. Some of the iron may be stored directly within the enterocyte as ferritin, while other iron will be released from the cell to end up bound to blood transferrin [8].

2. Intracellular iron trafficking and transportation

There is only one known cellular iron exporter, namely ferroportin [9–11]. This protein is found on the basolateral membrane of enterocytes as well as on other cells such as reticuloendothelial macrophages that export recycled iron, hepatocytes that release storage iron and on differentiating erythrocytes. Ferroportin exports iron in the ferrous (Fe^{2+}) form, but transferrin binds iron in the ferric (Fe^{3+}) form, so ferroxidases are believed to play a role in iron export. In intestinal enterocytes it is believed that hephaestin is the active ferroxidase, while in other cells this action is performed by the either circulating or GPI-linked multicopper ferroxidase ceruloplasmin [3,5]. Once bound to transferrin the iron is delivered to peripheral tissues by the transferrin–transferrin receptor system. After binding to the transferrin receptor, transferrin is internalized by receptor mediated endocytosis and upon acidification of the endosome iron is released from transferrin and converted to the ferrous form (Fe^{2+}) by the ferrireductase Steap family proteins [12,13] and transported across the membrane of the endosome into the cytoplasm by the action of the ferrous iron transporter divalent metal ion transporter 1 (DMT-1) protein [3,5].

Ferroportin has been shown to be regulated transcriptionally in enterocytes and macrophages [11,14] and to be translationally regulated by the iron responsive element (IRE) present in the 5′-UTR of the ferroportin mRNA through the action of iron regulatory proteins (IRP). The IRE–IRPs system is controlled by intracellular iron levels [9,11,15,16]. IRPs are activated during low iron condition under which they bind to the IRE of ferroportin mRNA resulting in translational suppression. Restrained ferroportin expression leads to reduced iron export, maintaining iron for cellular requirements. In addition, ferroportin is regulated at a post-translational step by the master iron homeostasis hormone, hepcidin. In erythroid precursor cells (and in enterocytes) a second mRNA encoding for ferroportin has been reported [17,18]. This mRNA is produced by the use of an alternate, upstream gene promoter and has an identical open reading frame in the mRNA, and as such the protein produced is identical. Critically, this second mRNA (termed FPN1B) does not contain an IRE in the 5′-UTR, and as such is not regulated by iron deficit [18]. It is currently believed that during erythropoiesis the relative expression of these two messages is coordinated to ensure that iron is exported from the cells during early differentiation, but kept in the cells during late differentiation when heme synthesis begins and iron demand is at its highest [18].

2.1. Iron regulation by hepcidin

The absorption of iron by enterocytes, the efflux of recycled iron from macrophages and the efflux of stored iron by

hepatocytes are all systemically controlled by the 25 amino acid peptide hormone hepcidin [19,20] which is produced predominantly in hepatocytes. Hepcidin is initially synthesized as an 84 amino acid preprohormone, before undergoing processing to generate a 60 amino acid prohormone and finally a 25 amino acid hormone [19]. The structure of the mature hormone is a compactly folded protein with 32% beta-sheet and 4 disulphide bonds [19]. Hepcidin regulates iron efflux by post-translationally negatively regulating ferroportin, the sole iron efflux channel. When iron is present in the plasma in excess hepcidin is secreted from the liver into the plasma. Hepcidin then directly binds to ferroportin expressed on the surface of iron storage cells, triggering endocytosis of both ferroportin and hepcidin which are subsequently degraded by lysosomes [21]. A reduction of ferroportin expression on the cell surface results in less intracellular iron being exported from storage cells, effectively locking iron inside the cell. This event reduces iron efflux into the plasma, returning iron to regular levels. The mechanism by which hepcidin regulates absorption of dietary iron is less clear as ferroportin is located on the basal surface of enterocytes cells, while absorption of dietary iron occurs on the apical surface. The mechanism by which a reduction in the basolateral ferroportin is communicated to the apical iron absorption mechanism remains unclear, although rising intracellular iron levels (as a consequence of reduced efflux) may play a role [5,21]. Mechanistically however it is known that hepcidin inhibits the uptake step of duodenal iron absorption but does not affect the proportion of iron transferred to the circulation [22].

2.2. Regulation of hepcidin expression

Hepcidin is primarily produced by hepatocytes [21], and hepcidin production is regulated by a number of factors, although it is believed that the primary stimuli are the level of iron in the plasma. Some studies have suggested that the two transferrin receptors (TfR1 and TfR2) together with the membrane protein hereditary hemochromatosis protein (*HFE*) serve to sense iron levels in the body and induce hepcidin expression, but the mechanism is incompletely understood [5]. A second pathway, the bone morphogenic protein (BMP) pathway, is probably activated in response to iron levels in intracellular iron stores, which results in increased expression of BMP6 which binds to a BMP receptor (BMPR) in association with a co-receptor hemojuvelin which activates the Smad signaling pathway resulting in increased hepcidin expression [5]. Recent evidence has shown that TMPRSS6, which encodes a hepatocyte-specific type II transmembrane serine protease, matriptase-2, cleaves hemojuvelin decreasing the BMP-SMAD signaling axis, and thus inhibiting hepcidin expression [23]. Inhibition of the expression of this inhibitory protein may provide an attractive pathway for increasing hepcidin expression in beta-thalassemia patients [24], although some drawbacks exist [25]. A further hepcidin stimuli is inflammation, and this pathway is mainly modulated through the inflammatory cytokine IL-6 which activates the JAK-STAT3 pathway leading to increased hepcidin expression in the liver [26,27].

To maintain iron homeostasis, the negative regulation of hepcidin expression is an important mechanism to ensure the availability of iron for biological activities. The erythropoietic cells of the bone marrow are the main consumers of iron provided by transferrin, and erythropoiesis is wholly dependent upon this source of iron. During erythropoiesis, erythroid cells secrete a factor or factors that suppress hepcidin expression in liver cells. This results in increased ferroportin activity and the transfer of iron from cellular stores to transferrin, thus supplying the demand for iron during erythropoiesis. The suppression of hepcidin also increases absorption of dietary iron [22]. The factor or factors secreted by erythroid cells remain to be clearly elucidated. Studies have implicated the growth and differentiation factor 15 (GDF15) which is known to be highly elevated in beta-thalassemia patients [28]. Other studies have suggested that the twisted gastrulation protein (TWSG1) may be the primary factor regulating hepcidin suppression in liver cells [29]. Moreover, erythroferrone has been recently identified and proposed as a candidate for suppressing hepcidin expression during erythropoiesis [30].

3. Thalassemia syndromes

Thalassemia syndromes are a group of inherited hematological disorders that constitute a major public health problems worldwide [31]. The term “thalassemia”, which has been used to describe autosomal recessive anemic disorders, is derived from the Greek word “thalassa” (the Mediterranean sea) and “haima” (blood) since it was first applied to the anemias frequently encountered in people around the Mediterranean sea, particularly in Italy and the Greek coast and nearby islands [32]. Thalassemia syndromes are a heterogeneous group of anemias which are caused by genetic defects in globin genes. Defect of one or more globin genes cause a partial reduction or total depletion of globin chains synthesis thereby leading to inadequate production of hemoglobin [33]. The major types of thalassemia are alpha- and beta-thalassemia which are classified according to the nature of the defective globin [33].

4. Beta-thalassemia

Beta-thalassemia is a heterogeneous group of disorders leading to decreased or absent beta-globin production. A genetic defect of one or two beta-globin genes, which are located on chromosome 11 (p15.5), is the cause of beta-thalassemia [34]. To date, more than 200 point mutations have been identified in beta-globin genes and the immediate flanking regions [35]. The expression of mutated beta-globin genes can result in reduced or absent beta-globin production, unlike the large gene deletions in alpha-globin gene, which solely result in loss of function [34]. According to these finding, beta-thalassemia can be phenotypically classified into 2 types; $beta^0$-thalassemia where no beta-globin chains are synthesized and $beta^+$-thalassemia where some beta-globin chains are synthesized [36]. In $beta^+$-thalassemia, there is a 5% to 30% reduction of beta-globin chains from normal levels [37]. The hallmark of beta-thalassemia is the reduced production or absence of Hb A ($alpha_2$ $beta_2$), reactivation of Hb F ($alpha_2gamma_2$), and importantly, accumulation of excess alpha-globin chains which appears to underlie the main physiopathology of the disease. Patients with the most severe form of beta-thalassemia ($beta^0$/$beta^0$) develop serious microcytic anemia due to severe hemolysis and impaired production of new RBCs [38]. Bone deformity

as a result of erythroid hyperplasia and enlargement of liver and spleen are also observed [35]. In heterozygous beta-thalassemia (beta/beta^0, beta/beta$^+$) or beta-thalassemia traits, Hb A formation is substantial due to the output from the remaining intact beta-globin gene, thus resulting in a nearly asymptomatic presentation with mild hypochromia with microcytosis but with relatively little evidence of anemia, hemolysis, or impaired erythropoiesis.

The most common subgroup of beta-thalassemia is beta-thalassemia/Hb E which accounts for almost 50% of the patients with severe beta-thalassemia worldwide [38]. This compound heterozygote is very common in many regions where Hb E is predominant. Hemoglobin E (Hb E) is the structural hemoglobin variant which is the most common in Southeast Asia including Thailand (10–50% of the population) [39,40]. The betaE-globin gene produces only small amounts of betaE-globin chains, which is similar to some mutations causing beta$^+$-thalassemia and therefore Hb E trait resembles a very mild beta$^+$-thalassemia trait while Hb E homozygotes exhibit more microcytosis but are still asymptomatic [41]. The severity of beta-thalassemia/Hb E generally depends on the co-inheritance of alpha-globin hemoglobinopathies as well as the level of Hb F. Although patients with beta$^+$-thalassemia/Hb E develop a mild anemia with only a few clinical abnormalities, an extraordinarily wide clinical spectrum, ranging from a moderate to a severe form of anemia resembling homozygous beta^0-thalassemia are observed in beta^0-thalassemia/Hb E patients [40,42–44].

4.1. Molecular pathogenesis of beta-thalassemia

The main pathophysiology of beta-thalassemia is caused by the unbalanced production of alpha-globin and βετα-globin chains where alpha-globin chains appear to be in excess [35]. Unlike beta-globin chains, alpha-globin chains are unable to form stable tetramers thus free excess alpha-globin chains tend to form insoluble aggregates which precipitate within the developing erythroid cell. This results in the induction of apoptosis in the developing erythroid precursor at the polychromatophilic normoblast stage in a process termed ineffective erythropoiesis [45]. In the small percentage of erythroid cells that progress to maturation, the accumulation of free alpha-globin chains efficiently generates ROS and oxidative stress, resulting in RBC membrane damage and subsequently increased hemolysis [46]. In normal RBCs hemoglobin is reversibly oxidized to methemoglobin, with cytochrome b5 reductase mediating the reduction back to hemoglobin. However, free globin chains (both alpha and beta) are susceptible to oxidation to hemichromes which can become irreversibly modified [47], allowing the hemichrome iron to generate reactive oxygen species [48]. A large part of the difference in pathology between αλπηα- and βετα-thalassemia arises from the fact that the excess βετα-chains present in αλπηα-thalassemia can form a soluble tetramer (hemoglobin H, HbH) while the excess αλπηα-globin chains present in beta-thalassemia cannot, resulting in the deposition of insoluble aggregates in the RBC membranes. In beta-thalassemia therefore, the combination of ineffective erythropoiesis of the developing erythroid precursor cells and increased hemolysis of the mature RBC are the main causes of anemia in these patients. The anemia in these patients leads to a feedback loop that results in increased expansion of erythroid progenitors and accelerated erythroid differentiation [49]. The markedly increased erythropoiesis in beta-thalassemia has been reported in many studies either by prediction, based on ferrokinetic studies [50], by differential counting from bone marrow aspirates [51], or by *in vitro* observation of CD34 hematopoietic progenitor culture [45,52–54]. The marked expansion of the erythroid mass is a well documented feature of intermediate and severe beta-thalassemia cases which results in the generation of more distressing features such as organ enlargement and particularly bone deformity and fragility [42,55,56]. Ultrastructural studies using electron microscopy have shown that the precipitated αλπηα-globin chains are in both the cytoplasm and the nucleus [57] and begin to be present predominately in polychromatic erythroblasts [58–60]. Moreover, abnormal erythroid nuclei showing a partial loss of nuclear membrane and presence of intranuclear aggregates of αλπηα-globin chains have also been observed in bone marrow erythroblasts of homozygous βετα-thalassemia patients [58]. These findings led to investigations of intramedullary death which showed later that programmed cell death or apoptosis clearly occurred in erythroid precursors of beta-thalassemia major as detected by DNA ladder formation [61] and the outer externalization of phosphatidylserine (PS) to the membrane leaflet [62]. Additionally, *in vitro* studies have also demonstrated that apoptosis primarily occurs at the polychromatophilic normoblast stage [45], the intermediate stage of erythroid precursor differentiation where the αλπηα-globin chain aggregates appear to present [58]. Previous studies have demonstrated that heat shock protein 70 (HSP70) interacts directly with excess free αλπηα-globin chains and is sequestered in the cytoplasm [63]. This prevents HSP70 from performing its normal physiological role of protecting GATA-binding factor 1 (GATA1) from proteolytic cleavage resulting in premature degradation of GATA1 and maturation arrest and apoptosis of polychromatic normoblasts [64]. The marked degree of anemia, due to ineffective erythropoiesis, combined with a considerable tissue hypoxia promote erythropoietin (EPO) production, which has been shown in several studies to be increased in beta-thalassemia/Hb E patients as compared to normal controls [52,65–67]. Increased levels of EPO are believed to be the main factor driving the expansion of the erythroid mass. Extensive erythropoiesis induces a large erythropoietic mass which can be found in the bone marrow, liver and spleen, as well as at extramedullary sites.

4.2. Iron overload in beta-thalassemia

Severe cases of beta-thalassemia require regular blood transfusion to reduce the chronic anemia. Multiple blood transfusions, increased hemolysis of red blood cells and increased gastrointestinal iron absorption (Figure 1) lead to iron overload [68], and cardiomyopathy as a consequence of iron overloading is the most common cause of death in transfusion-dependent thalassemia patients [69]. The human body loses only 1–2 mg iron per day, while a unit of transfused red blood cells contains approximately 200 mg of iron [38]. A patient who receives 25 units of blood per year, accumulates 5 g of iron each year in the absence of any iron chelation therapy. Excess iron is extremely toxic to all cells of the body and can cause serious and irreversible organic damage, such as cirrhosis, diabetes, heart disease, and hypogonadism which lead to significant morbidity and mortality if untreated [68]. The iron overload on the body can be estimated by means of serum

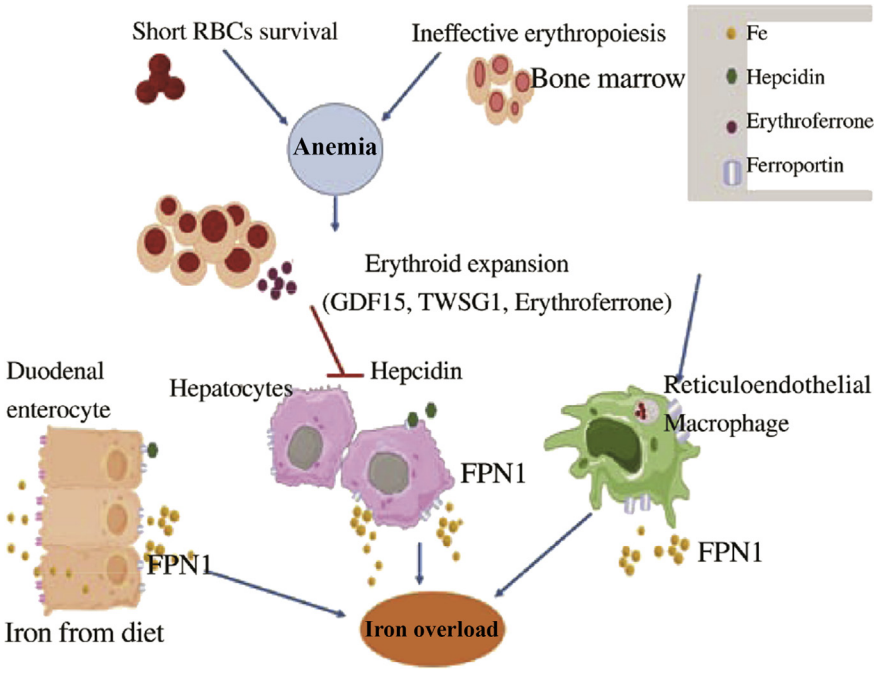

Figure 1. Proposed mechanism of iron dysregulation in beta-thalassemia disease.
Beta-thalassemia patients experience anemia mainly from ineffective erythropoiesis and shortened red blood cell (RBC) survival. The anemia induces erythropoietin (EPO) production leading to enhanced erythropoiesis. The dramatically increased erythroid expansion activates secretion of erythroid factors including growth differentiation factor 15 (GDF15), twisted-gastrulation 1 (TWSG1) and erythroferrone (ERFE). Excessive erythroid factors suppress hepcidin expression in hepatocytes resulting in increased iron absorption from duodenal enterocytes, the release of iron from the liver and the reticuloendothelial system, culminating in iron overload.

ferritin, hepatic iron concentration, urinary iron excretion and TIBC levels [70]. Threshold values for iron toxicity are a liver iron concentration exceeding 440 mmol/g dry weight, serum ferritin >2500 ng/mL, urinary iron excretion >20 mg/day, and transferrin saturation >75% [71]. The estimation of hepatic iron concentration by magnetic resonance imaging (MRI) is the most commonly employed test to evaluate iron overload in thalassemia major [72]. Increased iron overload has also been reported in patients with non-transfusion dependent thalassemia (NTDT) [73]. beta-thalassemia carriers and patients who have a histidine to aspartic acid (H63D) mutation at codon 63 of the *HFE* gene show iron overloading, suggesting that the H63D mutation may have a modulating effect on iron absorption [74,75].

4.2.1. Pathophysiology of iron overload in beta-thalassemia

The excess iron in beta-thalassemia patients saturates the ability of the transferrin iron transport system, leading to non-transferrin bound iron (NTBI) and labile plasma iron (LPI) starting to circulate in plasma and subsequently becoming deposited inside susceptible cells [71]. Rather than using the transferrin receptor, NTBI enters cells by other cellular channels including L-type voltage-dependent Ca^{2+} channel (LVDCC), a promiscuous divalent cation transporter [76] and Zip14, a member of the SLC39A zinc transporter family [77]. Long-term uptake and accumulation of NTBI and LIP, its redox active component, leads to increased levels of storage iron and labile cellular iron [78]. Tissues susceptible to iron accumulation by this mechanism include the liver, endocrine system and myocardium [79]. When the magnitude of the cellular labile iron pool exceeds the capacity of the cell to synthesize new ferritin molecules, a critical concentration is reached that can generate reactive oxygen species (ROS). ROS produced by the metabolism of NTBI plays a central role in inducing cellular dysfunction, apoptosis, and necrosis [80]. A variety of ROS, most notably hydroxyl radicals, increase lipid peroxidation and organelle damage, leading to cell death and fibrogenesis mediated by transforming growth factor beta1 (TGF-beta1) [81]. An underappreciated effect of iron overload is increased infection risk that is a high cause of mortality in beta-thalassemia patients [82]. Oxidative stress is a major inducer of autophagy, which is important in the removal of oxidized proteins and damaged mitochondria. The increased activation of autophagy has been reported in beta-thalassemia/Hb E erythroblasts as compared to normal control erythroblasts [83], suggesting that high levels of autophagy in beta-thalassemia/Hb E erythroblasts might be induced by ROS that contribute to the increased levels of apoptosis that lead to ineffective erythropoiesis in beta-thalassemia/Hb E erythroblasts [83].

Recently, dysregulation of ferroportin *mRNA* has been reported in beta-thalassemia/Hb E. While erythroblasts from normal controls show increased expression of ferroportin expression during differentiation under iron overload conditions, erythroblasts from beta-thalassemia/Hb E patients show no increase in ferroportin expression under the same growth conditions [84]. Thus the ability of these critical erythroid cells to export excess iron is curtailed, adding to the direct biological consequences of iron overload.

Iron overload can also contribute to ineffective erythropoiesis to a varying extent depending on the disorder (Figure 1). It has been suggested that the production of growth differentiation factor 15 (GDF15) [28,85] and possibly other proteins, such as twisted-gastrulation 1 (TWSG1) [29], contributes to the inhibition of hepcidin synthesis and thus promotes iron

absorption in beta-thalassemia patients. Kautz and colleagues suggested that, upon increased erythropoiesis, bone marrow and spleen erythroblasts increasingly produce erythroferrone, which, upon secretion into the circulation, directly acts on the liver to inhibit hepcidin production and mediates increased iron mobilization and absorption during periods of erythropoietic stress [30].

4.3. Hepcidin expression in beta-thalassemia

Deficits in hepcidin expression in relation to thalassemia were first reported in a mouse model system (C57BI/6 $Hbb^{th3/+}$) of severe anemia [86], and since then hepcidin levels in beta-thalassemia/Hb E disease, beta-thalassemia trait and Hb E trait carriers have been reported [87]. Levels of hepcidin in beta-thalassemia patients have been shown to be extremely low [88–91] and serum from beta-thalassemia patients has been shown to suppress hepcidin expression in liver cells [28]. This will lead to continual, effectively unregulated absorption of dietary iron, leading to overloading. Liver hepcidin mRNA expression in patients with thalassemia major and thalassemia intermedia is inversely correlated with soluble transferrin receptor (sTfR) and erythropoietin (EPO), but not with iron stores [92]. The study proposed that hepcidin suppression in beta-thalassemia/Hb disease is associated with iron loading, saturation of iron binding proteins, and consequently, organ damage as indicated by an inverse association between hepcidin and NTBI across all patients, as well as correlation of NTBI and ferritin or LIC, and correlation of iron loading with ALT, an enzymatic marker of hepatic damage [87]. While moderate suppression of hepcidin with enhanced iron absorption was also observed in beta-thalassemia carriers; this was not the case with Hb E trait carriers [87,93]. The coinheritance of alpha-thalassemia results in a reduction in erythropoiesis and ameliorates hepcidin suppression [87]. Less severe forms of ineffective erythropoiesis, as observed in alpha-thalassemia, may cause late-onset and milder iron overload.

4.4. Hepcidin in the pathogenesis of beta-thalassemia

Anemia, tissue hypoxia and increased EPO production observed in beta-thalassemia promote the suppression of hepcidin and increase iron absorption in response to the demand for iron by erythroblasts [94]. Several hepcidin inhibitors released from erythroblasts during the process of differentiation have been proposed to regulate hepcidin expression in beta-thalassemia. The cytokine members of the TGF-beta family, namely growth differentiation factor 15 (GDF15) was shown to be up-regulated in serum from thalassemia patients and can suppress hepcidin expression in hepatoma cells or in isolated human hepatocytes [28,95]. Serum levels of this cytokine are strikingly elevated in patients with homozygous beta-thalassemia, while intermediate levels are found in carriers of alpha-thalassemia and in beta-thalassemia trait carriers. In contrast, sickle cell patients whose anemia is related to chronic hemolysis rather than ineffective erythropoiesis, show no or only modest GDF15 elevation [28]. TWSG1 is a second erythroid factor that has been identified as a hepcidin regulator. Levels of this protein are increased in the bone marrow, spleen and liver of thalassemic mice [29]. However, the level of TWSG1 in the serum of thalassemia patients remains to be reported. Hepcidin inhibition by the liver serine protease TMPRSS6 has also been shown [23,96]. More recently, the hormone erythroferrone (ERFE) has been identified as a new erythroid regulator of hepcidin synthesis [30,97]. In mouse models of intermediate thalassemia, bone marrow ERFE expression is increased in response to erythropoietin and mediates hepcidin suppression during stress erythropoiesis. ERFE-deficient mice fail to suppress hepcidin rapidly after hemorrhage and mice exhibit a delay in recovery from blood loss [97]. However, the molecular mechanisms underlying the suppression of hepcidin by these erythroid factors, as well as the interplay between the factors remain to be clarified.

4.5. Therapeutic targeting of hepcidin in beta-thalassemia

The standard treatment of severe beta-thalassemia is currently based on blood transfusions, iron chelation and splenectomy, allowing an increased survival and amelioration of the patients' quality of life [98]. A cure for inherited beta-thalassemia can be achieved by allogeneic hematopoietic stem cell transplantation, but the need to control transplant-related complications and the requirement for matched donors make this option available to only some patients [99], and as many as 60% of patients lack a suitable donor [99]. Alternative treatments, such as gene therapy or the induction of fetal hemoglobin (Hb F) are promising [100], but have yet to make it to the bedside. As iron overload is the most important complication for the patients with blood transfusion, iron chelation is essential to control iron overload and its toxicity [101] and effective management of iron overload in thalassemia requires monitoring both for iron toxicity and the effects of excessive chelation. Recently however, improved knowledge of the relationships between iron overload and hepcidin has led to the development of novel approaches that target the pathophysiology of the disease with the aim of reducing iron overload and, at the same time, of alleviating ineffective erythropoiesis. Hepcidin levels are low in thalassemia patients with concomitant pathophysiological consequences, and the restoration of hepcidin to normal levels is an attractive novel therapeutic strategy. Studies in a mouse model of beta-thalassemia have shown that increasing hepcidin reduces iron bioavailable to erythroblasts, resulting in decreased heme synthesis and improved erythroid precursor and reticulocyte survival [102]. Similarly small synthetic peptides (minihepcidins) can decrease serum iron, prevent iron overload and promote iron redistribution in hepcidin-deficient mice [103]. In another approach, administration of BMP6, the natural ligand of the BMP receptor involved in hepcidin regulation, was shown to activate the hepcidin transcription regulated pathway and to correct the high iron saturation and iron maldistribution in a *HFE* model of hereditary hemochromatosis [104]. Transgenic inactivation of the membrane protease TMPRSS6 in *HFE* mice increased hepcidin expression and reversed their iron overload phenotype, suggesting that the administration of a specific inhibitor of the enzymatic activity of TMPRSS6 could be used to treat iron overload [105,106]. An RNAi therapeutic targeting Tmprss6 has been shown to decrease iron overload with diminished hepcidin expression and may have efficacy in modifying disease-associated morbidities of thalassemia [24]. A combination therapy of RNAi against Tmprss6 together with

administration of the iron chelator deferiprone has been reported to result in a significant reduction of liver iron content and improved erythropoietic efficiency in thalassemic mice [107,108].

5. Conclusions

While the genetic lesions engendering beta-thalassemia are well characterized, how these lesions lead to the full pathological spectrum of the disease remain less well understood. While deposition of unpaired αλπηα-globin chains during erythropoiesis is a major event, it is clear that dysregulation of iron homeostasis in both transfusion dependent and transfusion independent beta-thalassemia patients is a dominant physiological effect. Understanding hepcidin expression and regulation in the context of the beta-thalassemia patient is vital to developing rational therapeutic interventions to provide safe, effective and lifelong treatments.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Hypermethylation of 28S ribosomal RNA in β-thalassemia trait carriers

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ABSTRACT

Ribosome biogenesis is the process of synthesis of the cellular ribosomes which mediate protein translation. Integral with the ribosomes are four cytoplasmic ribosomal RNAs (rRNAs) which show extensive post-transcriptional modifications including 2′-*O*-methylation and pseudouridylation. Several hereditary hematologic diseases including Diamond-Blackfan anemia have been shown to be associated with defects in ribosome biogenesis. Thalassemia is the most important hematologic inherited genetic disease worldwide, and this study examined the post-transcriptional ribose methylation status of three specific active sites of the 28S rRNA molecule at positions 1858, 4197 and 4506 of β-thalassemia trait carriers and normal controls. Samples from whole blood and cultured erythroid cells were examined. Results showed that site 4506 was hypermethylated in β-thalassemia trait carriers in both cohorts. Expression of fibrillarin, the ribosomal RNA methyltransferase as well as snoRNAs were additionally quantified by RT-qPCR and evidence of dysregulation was seen. Hemoglobin E trait carriers also showed evidence of dysregulation. These results provide the first evidence that ribosome biogenesis is dysregulated in β-thalassemia trait carriers.

1. Introduction

β-thalassemia refers to the hereditary blood disorder characterized by reduced (β^+) or absent (β^0) beta globin chains and more than 200 different point mutations in addition to cases of gross gene deletions have been characterized to date as being associated with β-thalassemia [22]. The reduced or absent expression of the β-globin chain leads to a loss of balanced globin chain synthesis and the excess α-globin chains precipitate in the cell resulting in ineffective erythropoiesis and increased hemolysis [15]. Collectively the loss of functional hemoglobin presents as an anemia of variable severity that is dependent upon a number of factors including the co-inheritance of α-globinopathies [20]. Heterozygous β-thalassemia generally presents as an asymptomatic to intermediate phenotype, while compound heterozygote β^0-thalassemia typically present as a severe, transfusion dependent condition [22].

Mature red blood cells arise from hematopoetic stem cells predominantly resident in the bone marrow in a process that is controlled by the action of a number of cytokines including erythropoietin (EPO). In β-thalassemia the developmental process is believed to be inappropriately terminated by the induction of apoptosis as a consequence of precipitation of the unpaired α-globin chains in the process termed ineffective erythropoiesis [15]. The resulting anemia promotes the increased production of EPO, leading to expansion of the bone marrow mass [3]. Critically however, some evidence has suggested that erythroid progenitor cells in β-

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thalassemia patients may have an inherently increased expansion capacity, modulated at least in part by the hyperphosphorylation of ERK1/2 [21], a protein that regulates the expression of a large number of genes both directly and indirectly. Studies have shown that ERK1/2 plays a role in enhancing Myc activity by increasing stabilization of Myc through phosphorylation of Ser 62 resulting in a decrease of Myc ubiquitin mediated degradation [16]. The Myc family of transcription factors regulate the expression of thousands of genes that control a wide range of cellular processes including transcription, translation, regulation of chromatin structure, DNA replication, and more recently, ribosome biogenesis [18].

Ribosome biogenesis involves more than 170 accessory factors and involves a number of transcriptional and post-transcriptional events [4]. Mature ribosomes consist of four different ribosomal RNA (rRNA) molecules together with some 80 different ribosomal proteins, and the rRNA molecules undergo extensive co- and post-transciptional modification including endo and exo-nuclease cleavage, as well as specific 2′-*O*-methylation and pseudouridylation [1]. 2′-*O*-methylation is regulated by box C/D snoRNAs (small nucleolar RNAs) in association with fibrillarin, while box H/ACA snoRNAs in association with dyskerin regulate pseudouridylation [7]. These biochemical modifications could finely tune the catalytic ribozyme activity of the rRNAs which directly control protein synthesis [1]. Indeed, evidence is emerging that these post-transcriptional modifications serve to modulate ribosomal assembly and translational capacity [24].

Defects in ribosome biogenesis have been associated with several inherited bone marrow failure syndromes including Diamond-Blackfan anemia [12] which has been shown to result from mutations in a number of ribosomal protein genes [19]. Given that β-thalassemia is the most common anemia worldwide, we sought to determine if there was evidence of altered ribosome biogenesis in β-thalassemia and additionally looked at hemoglobin E trait.

2. Design and methods

2.1. Sample collection and preparation

In this study, all blood samples were taken after obtaining individual informed consent under a protocol approved by the Committee for Human Rights related to Experimentation, Mahidol University. For experiments utilizing whole blood, peripheral blood was taken from healthy normal (2.5 ml) and heterozygous β-thalassemia donors (0.5–1.5 ml). All individuals donating specimens used as whole blood were screened as heterozygous β-thalassemia as previously described [8]. Normal controls were chosen on normal HbA2 levels using the precise Bio-Rad Variant II Turbo cation-exchange HPLC assay (Bio-Rad Turbo) and by assessing normal hematologic parameters (HCT, RBC, HB, MCH, MCV). Genotyping data for these β-thalassemia trait carrier individuals is shown in Table 1. All samples for this part of the study were collected in evacuated tubes (PAXgene Blood RNA tubes; Qiagen, Hilden, GmbH, Germany) and mixed thoroughly with the RNA stabilizing agent provided in each tube before leaving them for 2 h at room temperature to allow the lysis of erythroid cells and RNA stabilization.

For experiments that cultured erythroid cells, 50 ml of peripheral blood was collected from normal controls, heterozygous β-thalassemia individuals and heterozygous Hb E individuals. Individuals donating blood for erythroid culture were screened by complete blood count (CBC) indices, hemoglobin typing and α-globin gene genotyping controls exactly as described previously [17].

2.2. Cell isolation and culture

CD34+ hematopoietic stem cells (HSCs) were isolated from peripheral blood and cultured under conditions driving erythropoiesis exactly as described elsewhere [17,21]. Briefly, peripheral blood mononuclear cells (PBMCs) were separated by overlaying on Lymphoprep reagent (Axis-Shield Poc AS, Oslo, Norway) and centrifugation at 600 × *g* for 20 min. $CD34^+$ HSCs were purified from isolated PBMCs using the human CD34 MicroBead kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to manufacturer's protocol. The cells were cultured in Iscove's Modified Dulbecco Medium (IMDM, Gibco BRL, Gaithesburg, MD) containing 15% human AB serum, 15% FBS (Gibco BRL), 20 ng/mL hSCF (Cell signaling technology, Danvers, MA), 2 U/mL EPO (Janssen-Cilag Ltd, Auckland, New Zealand) and 10 ng/mL IL-3 (Promokine, Heidelberg, Germany). Cells were grown and maintained at 37 °C with 5% CO_2. Media was replaced with fresh media without IL-3 on day 3 and day 7 of culture. Under these culture conditions on day 7 more than 95% of cells have proerythroblast morphology as previously established [21]. For this study, cells were cultured up to day 10, a time point at which globin gene expression is occurring as we have previously established [9].

2.3. RNA extraction and first strand cDNA synthesis

Total RNA was extracted from peripheral blood samples using the PAXgene Kit (Qiagen, Hilden, GmbH, Germany) while total RNA from day 10 erythroid cells was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. The concentration of total RNA from each sample was determined using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA). The purity of RNA was assessed by OD260/OD280 and the values ranged from 1.8 to 2.2. The integrity of extracted total RNA from erythroid cells was examined by separating 500 ng of each sample on 1% of non-denaturing agarose gels under RNAse free condition. The extracted total RNA derived from three colorectal cancer cells was used as the positive control of intact RNA profiles.

For analysis of 28S rRNA methylation, 5 ng of total RNA was used as a template for reverse transcription reaction and cDNA was synthesized using 5 μM of a specific reverse primer targeting to the downstream sequence of each methylation site (Table 2), 1× first strand buffer, 10 mM DTT, 200 U M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and either 10 μM or 1 mM dNTPs (Promega, Madison, WI) for low and high dNTPs reaction, respectively. Both low and high dNTPs samples were incubated at 37 °C for 50 min and the reaction was terminated by incubation at 70 °C for 15 min.

For analysis of fibrillarin gene expression of RNA from whole blood 100 ng total RNA was used as a template and cDNA was synthesized using 1 μM oligo dT in the presence of 2 μl of 10 mM dNTPs (Invitrogen, Carlsbad, CA), 100U/ul M-MLV reverse transcriptase. Samples were incubated at 42 °C for 90 min and the reaction was terminated by incubation at 70 °C for 15 min.

For analysis of fibrillarin, snoRNA, NOP56 and NOP58 gene expression of RNA from erythroid cells, 200 ng total RNA was used as template for RT-PCR and cDNA was synthesized using 5 μM random 6 mers, 2.5 μM oligo dT primer in the presence of 1× PrimeScript buffer and PrimeScript RT enzyme mix I (PrimeScript RT reagent kit; Takara Bio Inc., Shiga, Japan). Samples were incubated at 37 °C for 15 min and the reaction was terminated by incubation at 85 °C for 5 s.

2.4. qPCR and methylation analysis

Analysis of rRNA methylation status was undertaken as previously described [2] and standardization of reverse transcription

Table 1
Clinical parameters of heterozygous β-thalassemia patients.

Patient	Hb (g/l)	MCV (fl)	MCH (pg)	Genotype (mutation)[a] Usual name – HGVS nomenclature
1	no data	no data	no data	CD39_Beta0_C>T (HBB:c.118C>T) heterozygote
2	no data	no data	no data	−87_Beta+_C>G (HBB:c.-137C>G) heterozygote
3	no data	no data	no data	−29_Beta+_A>G (HBB:c.-79A>G) heterozygote
4	108	61	21	−88_Beta+_C>T (HBB:c.-138C>T) [b]
5	122	79	27	−29_Beta+_A>G (HBB:c.-79A>G) heterozygote
6	118	58	19	CD39_Beta0_C>T (HBB:c.118C>T) heterozygote
7	132	75	25	−87_Beta+_C>G (HBB:c.-137C>G) heterozygote
8	123	60	20	IVS1.1_Beta0_G>A (HBB:c.92+1G>A) heterozygote
9	122	79	25	−29_Beta+_A>G (HBB:c.-79A>G) heterozygote
10	no data	no data	no data	CD39_Beta0_C>T (HBB:c.118C>T) heterozygote
11	no data	no data	no data	CD6_Beta0_A>– (HBB:c.20delA) heterozygote
12	141	66	22	CD17_Beta0_A>T (HBB:c.52A>T) heterozygote
13	126	66	21	IVS1.110_Beta+_ G>A (HBB:c.93-21G>A) heterozygote
14	126	66	22	IVS1.110_Beta+_ G>A (HBB:c.93-21>A) heterozygote
15	124	79	26	−88_Beta+_C>T (HBB:c.-138C>T) heterozygote

[a] All mutations were found in the heterozygous state.
[b] Compound heterozygosity with the β^S mutation.

Table 2
Primer sequence for RT-PCR and qPCR.

Primer name	PCR reaction	Primer sequence
28S-A1858-RT	RT-PCR	5′-GATGAGCGTCGGCATCGGGC-3′
28S-U4197-RT	RT-PCR	5′-CCCTTCTGCTCCACGGGAGG-3′
28S-C4506-RT	RT-PCR	5′-CCATGGCAACAACACATCATCA-3′
28S-1858f	qPCR	5′-GTGGGCCACTTGGTAAGC-3′
28S-1858r	qPCR	5′-TTTCTGGGGTCTGATGAGC-3′
28S-4197f	qPCR	5′-CGGTACACCTGTCAAACG-3′
28S-4197r	qPCR	5′-CACGGGAGGTTTCTGTCCT-3′
28S-4506f	qPCR	5′- ATAGGGAACGTGAGCTGGGTTT-3′
28S-4506r	qPCR	5′-CCATGGCAACAACACATCATCA-3′
FBL-f	qPCR	5′-CCTGGGGAATCAGTTTATGG-3′
FBL-r	qPCR	5′-CCAGGCTCGGTACTCAATTT-3′
SNORD35A-f	qPCR	5′-TGATGTCCTTATCTCACGATGG-3′
SNORD35A-r	qPCR	5′-TGGCATCAGCTAAGCCATT-3′
SNORD35B-f	qPCR	5′-CCGGCATCAGTTTTACCAAG-3′
SNORD35B-r	qPCR	5′-TGATGTTTGTTTTCACGATGG-3′
SNORD38A-f	qPCR	5′-TCGTGATGAAAACTCTGTCCA-3′
SNORD38A-r	qPCR	5′-CCTCAGCCTAAAAGGCTCTC-3′
SNORD38B-f	qPCR	5′-TCTCCTCAGACACACTTTATCTTCA-3′
SNORD38B-r	qPCR	5′-TGAAAACTTTGTCCAGTTCTGCT-3′
NOP56-f	qPCR	5′-GAGGTGCCCACGAGTGTATT-3′
NOP56-r	qPCR	5′-TGCTTCCTTCATGACATCCA-3′
NOP58-f	qPCR	5′-TGATGGAGGGCAAAATCAAT-3′
NOP58-r	qPCR	5′-CGGTTCATGGGCTTCTTTTA-3′
GAPDH-f	qPCR	5′-AGCCACATCGCTCAGACAC-3′
GAPDH-r	qPCR	5′-GCCCAATACGACCAAATCC-3′

was achieved through addition of a synthetic polyA RNA (SmRNA) to each sample as described elsewhere [11]. The 45S pre-ribosomal RNA sequence (GenBank accession number NR_046235) was used as a template for design of methylation primers. All primers for methylation detection and other genes are given in Table 2. Quantitative PCR amplification of cDNA targets was carried out by using the LightCycler®480 SYBR green I master kit (Roche Diagnostics, Germany) and amplification undertaken in a Lightcycler480 Multiwell Plate 96 machine (Roche Diagnostics, Germany).

The specificity of PCR products was determined by a melting curve analysis immediately after the PCR amplification. The efficiency of PCR reaction was determined by amplifying GAPDH using serial dilutions of total RNA in parallel to the experimental samples. The methylation level of 28S rRNA was evaluated based on the difference of amplification cycle between high and low dNTPs sample of each methylation site by using $2^{-\Delta CT}$ method ($\Delta CT = Ct_{high} - Ct_{low}$). The relative gene expression of fibrillarin, SnoRNA, NOP56 and NOP58 was calculated by $2^{-\Delta\Delta CT}$ method ($\Delta\Delta CT = \Delta CT_{trait} - \Delta CT_{normal}$, $\Delta CT = Ct_{target} - Ct_{GAPDH}$).

2.5. Statistical analysis

All data from samples obtained from peripheral blood were analysed for statistical significance by the non-parametric Mann-Whitney *U* test using the XLStat software 2011.2.04 (MS Excel). The data of RNA samples from erythroid cells were analysed using two-independent sample *t*-test. All results are shown as the mean value with the standard error of deviation. Significance level was set at $P \leq 0.05$.

3. Results and discussion

The post transcriptional modification of rRNA molecules by specific 2′-*O*-methylation and pseudouridylation are critical steps in ribosome biogenesis [7]. Until recently, determination of the methylation status of rRNA molecules was a time consuming and technically difficult process [14]. The application of RT-qPCR with high and low concentration dNTPs to determine methylation status is a relatively new methodology that considerably simplifies determination of comparative methylation status of selected spe-

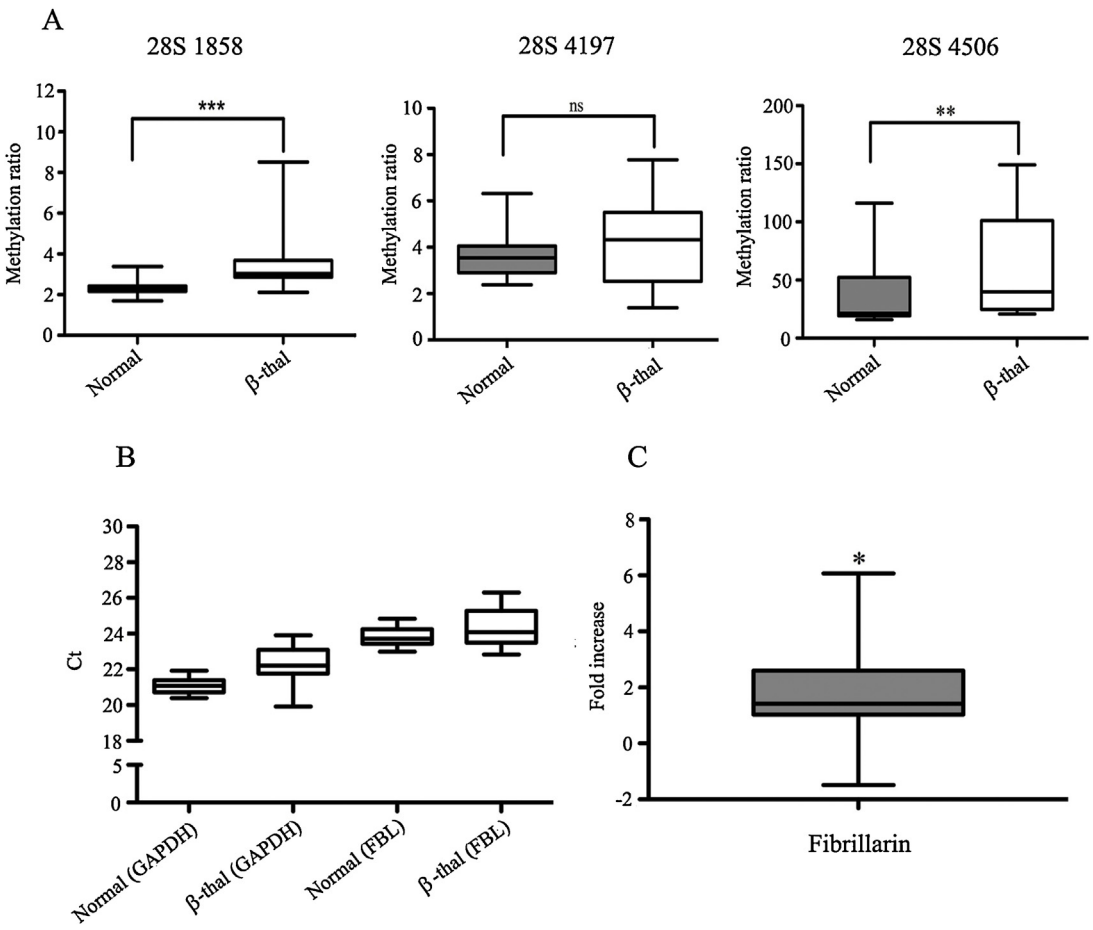

Fig. 1. Analysis of 28S rRNA methylation level and fibrillarin expression in peripheral blood from normal controls and heterozygous β-thalassemia. A. Graphical presentation of box-plot analysis from RT-qPCR of the specific methylated sites of 1858, 4197 and 4506 from 15 normal controls (Normal) and 15 heterozygous β-thalassemia (β-thal) calculated as the methylation ratio. Error bars represent S.D (*, $p < 0.05$). B. Graphical presentation of box-plot analysis of Ct values of GAPDH and fibrillarin in 15 normal controls and 15 heterozygous β-thalassemia. C. Box-plot analysis of relative fold increase of fibrillarin expression (heterozygous β-thalassemia over normal) examined by RT-qPCR normalized by GAPDH of 16 normal controls and 16 heterozygous β-thalassemia. Error bars represent S.D (*, $p < 0.05$).

cific residues [2]. The methylation status of three sites (1858, 4197 and 4506) in 28S rRNA extracted from whole blood was investigated in 18 normal controls and 15 β-thalassemia trait carriers by RT-qPCR. These sites were chosen for their localization within a key functional domain of the 28S rRNAs, *i.e.*, the peptidyl transferase center [5]. From quantitative PCR, melting curve analysis was used to confirm the specificity of the amplification products (See Supplemental Figs. 1–11). In addition, amplification cycle analysis verified that differentially amplified products were obtained from high and low dNTPs condition (See Supplemental Figs. 12–14). The difference in amplification was calculated and the results are expressed as the methylation ratio. The results showed that two of the sites, at positions 1858 and 4506 were significantly hypermethylated in trait carriers (3.45 ± 1.50 and 61.69 ± 45.39 respectively) as compared to normal control samples (2.33 ± 0.38 and 37.16 ± 30.95 respectively; $p = 0.0001$ and 0.0075 respectively) while the third site at position 4197 showed no significant difference in methylation ratio between normal controls and trait carriers (3.73 ± 1.01 and 4.16 ± 1.66, respectively; $p = 0.39$) as shown in Fig. 1A. However, it should be noted that there is a methylation site at position 4198, immediately following nucleotide 4197. As such, the methylation level derived from RT-qPCR is derived from both methylation sites, and they cannot be distinguished by the technique. However, as there is no significant difference in the methylation level at these positions in all sample groups, the lack of discrimination at this position is of minor significance.

As noted earlier 2′-*O*-methylation of rRNA is undertaken by box C/D snoRNAs in association with fibrillarin, which together with the Nop56, Nop58, and 15.5K/NHPX proteins form functional small nucleolar ribonuclear particles or snoRNP [7]. Given the evidence of hypermethylation of two sites of rRNA in β-thalassemia trait carriers, we further sought to determine whether expression of one of the components of the snoRNPs, fibrillarin, was also increased.

We therefore assessed the level of fibrillarin mRNA in total RNA from whole blood of 15 normal controls and 15 β-thalassemia trait carriers by RT-qPCR. We initially assessed the range of Ct of GAPDH and fibrillarin in both normal control samples and trait carriers to confirm that there was no significant variation of Ct in all samples examined (Fig. 1B). The result indicated that GAPDH gene is a suitable reference gene for RT-qPCR. Normalization of the data for fibrillarin against GAPDH showed a significantly higher level of fibrillarin expression in trait carriers as compared to normal controls (fold increase, 1.64 ± 2.08 and $p = 0.016$; see Fig. 1C). Specificity of the amplification reactions was again confirmed by a melting curve analysis (See Supplemental Figures).

Given that the original experiments were undertaken on RNA extracted from whole blood, the RNA would originate from a number of different cell types. To provide direct evidence of ribosome biogenesis dysfunction in the pathologically important erythroid cells, the 2′-*O*-methylation status of rRNA from cultured erythroid cells was determined. To provide more information on the involvement of ribosome biogenesis in other hemoglobinopathies,

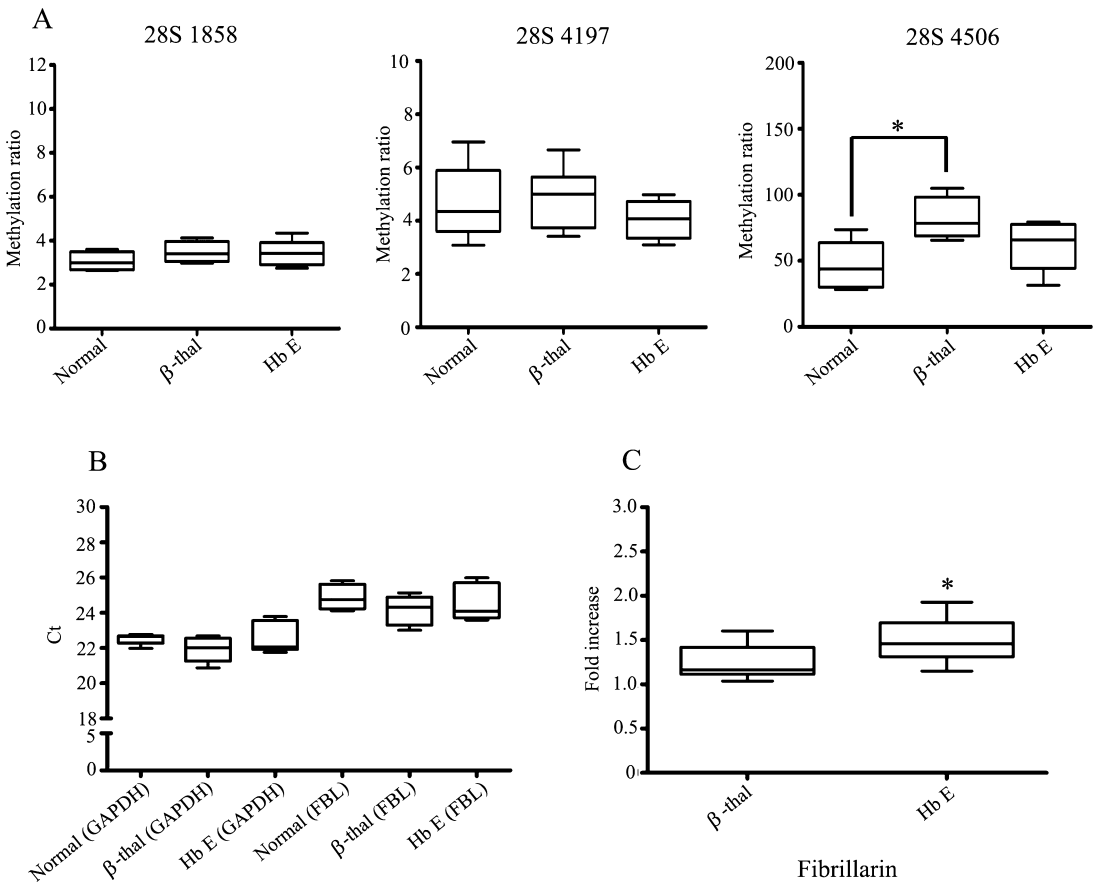

Fig. 2. Analysis of 28S rRNA methylation level and fibrillarin expression in erythroid cells. A. Graphical presentation of box-plot analysis from RT-qPCR of the specific methylated sites of 1858, 4197 and 4506 of day 10 erythroid cells derived from 3 normal controls (Normal), 3 heterozygous β-thalassemia (β-thal) and 3 heterozygous Hb E (Hb E) calculated as the methylation ratio from 2 independent experiments. Error bars represent S.D (*, $p < 0.05$). B. Graphical presentation of box-plot analysis of Ct values of GAPDH and fibrillarin from 3 independent experiments. C. Box-plot analysis of relative fold increase of fibrillarin expression (each sample group over normal) examined by RT-qPCR normalized by GAPDH of 3 normal controls. Error bars represent S.D (*, $p < 0.05$ and **, $p < 0.01$).

in addition to cells from β-thalassemia trait carriers, cells from hemoglobin E (HbE) trait carriers were also examined. CD34+ HSCs were therefore isolated and cultured to a time point after globin gene expression onset, namely day 10 of culture [9,23]. The integrity of extracted RNA was examined by non-denaturing agarose gel electrophoresis. From the RNA profile (See Supplemental Fig. 15), 28S and 18S rRNA of all samples were clearly separated when compared to RNA controls.

The methylation results (Fig. 2) showed that site 4506 was significantly hypermethylated in heterozygous β-thalassemia (81.86 ± 16.52) but not in heterozygous Hb E when compared to normal controls (46.29 ± 18.39, $p = 0.019$). The other 2 methylation sites examined at positions 4197 and 1858 showed no significant difference between any groups (Fig. 2). A slight increase in hypermethylation (3.48 ± 0.48 in heterozygous β-thalassemia compared with 3.05 ± 0.44 in normal controls) was observed at position 1858 which did not reach statistical significance, possibly reflecting the smaller sample size in this part of the study.

The expression of fibrillarin in erythroid cells was also determined and the results showed no significant variation of Ct of GAPDH and fibrillarin in samples (Fig. 2B). Normalization of fibrillarin expression against controls (Fig. 2C) showed that fibrillarin expression in erythroid cells derived from heterozygous Hb E trait carriers (1.51 ± 0.24, $p = 0.022$) was significantly up-regulated as compared to normal controls (1.05 ± 0.35), while heterozygous β-thalassemia trait carriers again showed a slight, but non-significant increase (1.25 ± 0.20).

To investigate the expression of other components of the snoRNPs complex, the expression level of specific box C/D snoRNA which are responsible for the methylation at significantly hypermethylated site was examined by RT-qPCR. The SNORD38A and SNORD38B guide the methylation at position 1858, while SNORD35A, SNORD35B guide the methylation at position 4506 [13]. From the result, the expression level of all snoRNA was significantly up-regulated in Hb E trait but not in β-thalassemia trait when compared to normal control (Fig. 3). In addition, NOP56 and NOP58 are associated with fibrillarin during pre-rRNA processing [6]. Thus, the expression of NOP56 and NOP58 mRNA was quantified. While no significant difference was seen in the expression of NOP56 mRNA, NOP58 mRNA was significantly up-regulated in both β-thalassemia trait (1.39 ± 0.24, $p = 0.02$) and Hb E trait (1.52 ± 0.38, $p = 0.012$) when compared to normal control (1.05 ± 0.32) (Fig. 3).

Two independent cohorts of β-thalassemia trait carriers, which for technical reasons were collected and evaluated four years apart, showed hypermethylation of 28S rRNA at position 4506. Evidence in both cohorts supports increased fibrillarin expression, although this did not reach significance in the erythroid cell experiment, possibly due to the small sample size. Additionally increased fibrillirin expression, together with increased expression of ancillary factors was observed in HbE trait carriers, suggesting that the alterations of ribosome biogenesis may be widespread in beta-hemoglobinopathies.

The data showing evidence of altered 2′-*O*-methylation of rRNA in 28S rRNA and increased levels of fibrillarin expression in β-thalassemia trait carriers together provide the first evidence of

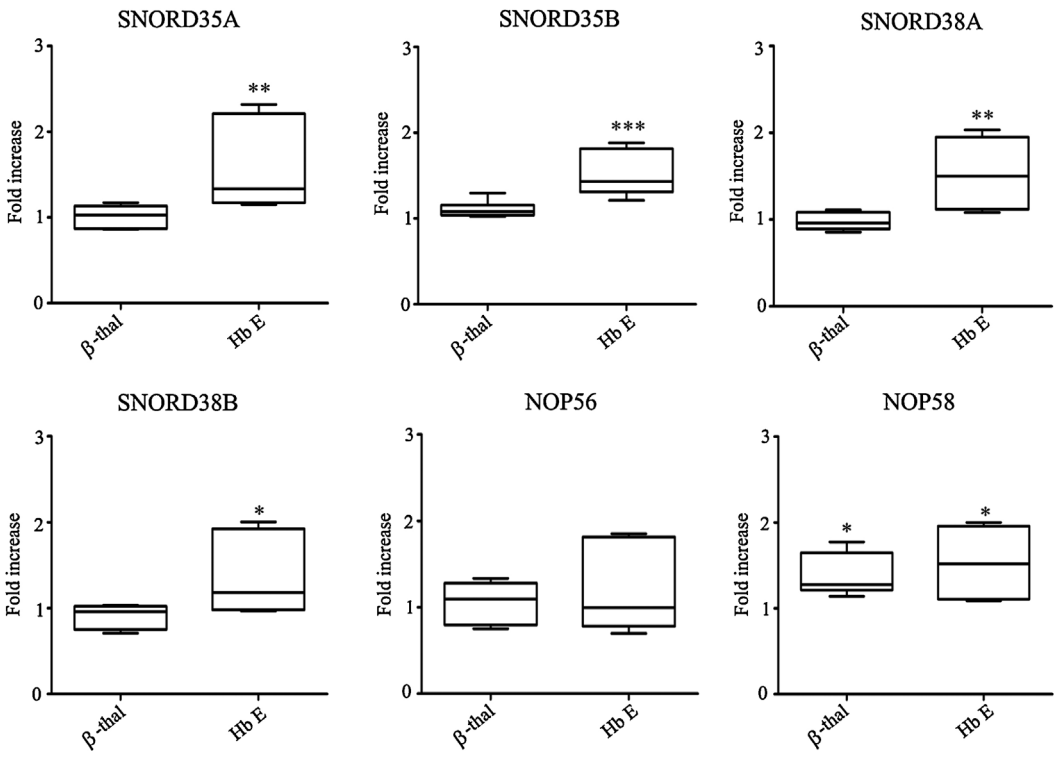

Fig. 3. Analysis of SnoRNA, NOP56 and NOP58 expression in erythroid cells. Graphical presentation of box-plot analysis of relative fold increase expression of SnoRNA; SNOD35A, SNORD35B, SNORD38A, SNORD38B and the associated snoRNPs complex NOP56 and NOP58 of day 10 erythroid cells derived from 3 normal controls (Normal), 3 heterozygous β-thalassemia (β-thal) and 3 heterozygous Hb E (Hb E). All expressions were examined by RT-qPCR normalized by GAPDH of 3 normal controls (each sample group over normal control). Error bars represent S.D (*, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$).

ribosome biogenesis dysfunction in these trait carriers. Defects in ribosome biogenesis have been associated with cancer and aging [10] as well as with inherited bone marrow failure syndromes [12] which have some parallels with β-thalassemia. Our results show that alteration in rRNA ribose methylation patterns could contribute to translational defects in thalassemia.

Authorship and disclosures

DRS and HCM designed the study. PJ, WS, SF and SS performed sample collection, genotyping and hemoglobin typing. PL and WS were responsible for all experimental work. DRS, HCM, JJD, SH, JE, PL, AF, and PJ were responsible for analysis and interpretation. DRS, WS HCM, JJD and PL wrote the manuscript with the approval from all authors. All authors declared no conflict of interest in this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2016.10.039.

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