Cardiac expression of HMOX1 and PGF in sickle cell mice and haem-treated wild type mice dominates organ expression profiles via Nrf2 (Nfe2l2)

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Summary
Haemolysis is a major feature of sickle cell disease (SCD) that contributes to organ damage. It is well established that haem, a product of haemolysis, induces expression of the enzyme that degrades it, haem oxygenase-1 (HMOX1). We have also shown that haem induces expression of placental growth factor (PGF), but the organ specificity of these responses has not been well-defined. As expected, we found high level expression of Hmox1 and Pgf transcripts in the reticuloendothelial system organs of transgenic sickle cell mice, but surprisingly strong expression in the heart (P < 0.0001). This pattern was largely replicated in wild type mice by intravenous injection of exogenous haem. In the heart, haem induced unexpectedly strong mRNA responses for Hmox1 (18-fold), Pgf (4-fold), and the haem transporter SLC48A1 (also termed Hrg1; 2.4-fold). This was comparable to the liver, the principal known haem-detoxifying organ. The NFE2L2 (also termed NRF2) transcription factor mediated much of the haem induction of Hmox1 and Hrg1 in all organs, but less so for Pgf. Our results indicate that the heart expresses haem response pathway genes at surprisingly high basal levels and shares with the liver a similar transcriptional response to circulating haem. The role of the heart in haem response should be investigated further.

Keywords: haemolysis, sickle cell disease, haem, Hmox1, Pgf.

Introduction
Advancements in medicine have contributed to a longer life span for patients with sickle cell disease (SCD), mainly in developed countries (Crocker et al, 1990), with increased development of age-related progressive end-organ injury, including kidney disease, pulmonary hypertension and diastolic heart failure. Even transplanted organs in patients with SCD have high risks of damage and failure circumstantially related to haemolysis and sickling (Huang et al, 2013; Kerins & Ooi, 2017).

Products of intravascular haemolysis contribute to impaired nitric oxide (NO) bioavailability, endothelial dysfunction and organ damage in SCD (Kato et al, 2007; Kato...
et al, 2017; Vichinsky, 2017). In addition, excess haem (iron protoporphyrin IX) released from damaged sickle red blood cells also acts as a damage-associated molecular pattern (DAMP), thereby activating inflammatory pathways (Ghosh et al, 2013; Belcher et al, 2014; Gladwin & Ofori-Acquah, 2014). Haem is well-documented to induce haem oxygenase-1 (HMOX1) in diverse cell types, a response that detoxifies haem (Vercellotti et al, 2016; Ingolia et al, 2017). Hmox1 generates equimolar amounts of carbon monoxide and bilirubin, which exerts antioxidant and anti-inflammatory effects (Wagener et al, 2001; Otterbein et al, 2003; Wagener et al, 2003). SLC48A1 (also termed HRG1), a haem transporter, is important for haem transport from the phagolysosome of macrophages during erythropagocytosis (Rajagopal et al, 2008; White et al, 2013). Hmox1 and Hrg1 expression is regulated by Nuclear factor (erythroid derived 2)-like 2 (NFE2L2, also termed NRF2), a key cytoprotective transcription factor that also regulates the basal and inducible expression of multiple antioxidant proteins and detoxification enzymes (Alam et al, 2003; Motohashi & Yamamoto, 2004).

Recent studies in mice have shown a protective effect of NRF2 in attenuating complications of SCD, such as inflammation, acute lung injury, anaemia (Ghosh et al, 2018; Ghosh et al, 2018) and spleen damage (Zhu et al, 2018).

Our laboratory has shown that haem also induces Placental Growth Factor (PGF) (Wang et al, 2014), which is an angiogenic growth factor secreted by proliferating erythroblasts during normal development (Tordjman et al, 2001). Pgf promotes the expression of the vasoconstrictor endothelin-1 by endothelial cells, associated with pulmonary hypertension in patients with SCD (Perelman et al, 2003; Sundaram et al, 2010) and thalassaemia intermedia (Kelaïdi et al, 2018). In non-haemolytic disease, such as chronic kidney disease, elevated PGF is associated with increased left ventricular mass index (Peiskerova et al, 2013) and other cardiovascular events (Heeschen et al, 2003; Matsui et al, 2015).

Organ damage in SCD is a multifactorial process and different organs may be affected in distinct ways by primary events, such as haemolysis and circulating non-haemoglobin bound haem. Because haemolysis contributes to organ damage in SCD, we investigated organ-specific expression patterns of Hmox1 and Pgf in sickle cell mice and modelled this in wild type mice by increasing circulating extracellular haem. Furthermore, we investigated the role of Nfe2l2 (also termed Nrf2) in the organ-specific transcriptional response pattern of Hmox1, Pgf and Hrg1.

Methods

Mice

Male and female Townes’ knocked-in transgenic sickle mouse (SS) and strain controls expressing normal human haemoglobin (AA mice), C57BL/6j (Nrf2+/+) and Nrf2−/− mice were used. C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA; stock number 000664) while SS, AA and Nrf2−/+ mice were obtained from a colony maintained by Dr Solomon Ofori-Acquah’s laboratory in our institution. Mouse genotypes were confirmed by polymerase chain reaction (PCR). Haem [Fe(III)PPIX, Sigma-Aldrich, St. Louis, MO] was first dissolved in 0.25 mol/l NaOH and then adjusted to pH 7.5 with HCl before filter sterilization. The haem solution was protected from light and injected into the tail vein of 14- to 16-week-old mice. The haemin dose was 50 µmol/kg body weight for SS and AA mice, and 120 µmol/kg body weight for Nrf2−/+ and Nrf2−/− mice. Some of the mice received sterile vehicle (0.25 mol/l NaOH adjusted to pH 7.5 with HCl used in preparation of haemin) as a control. A range of doses and times were tested; 3 h after injection produced consistent survival with no adverse effects on all strains of mice in this study.

Isolation of haematopoietic progenitors from bone marrow and spleen

Mouse bone marrow cells were isolated from the femur and tibia with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and 2 mmol/l EDTA from mice untreated and treated with vehicle or haemin for 3 h. The bone marrow suspension was filtered through a 70-µm-pore size cell strainer (Fisherbrand, Pittsburgh, PA, USA; Catalogue number 22363548) centrifuged at 300 g for 5 min. Splenocytes were separated from stromal elements by passing spleen tissue through 70-µm filters. The isolated splenocytes were incubated in 5 ml of red blood cell lysis buffer (155 mmol/l NH₄Cl, 14 mmol/l NaHCO₃ and 127 mmol/l EDTA) for 5 min at 8°C and washed twice with PBS.

Real-time PCR

Genes of interest (Table S1) were evaluated using the TaqMan® Gene expression assay and the TaqMan® RNA-to-Ct™ 1-Step Kit (both from ThermoFisher Scientific, Pittsburgh, PA, USA) according to the manufacturer’s instructions. Whole organs were harvested from mice immediately after death or 3 h after haemin injection. Freshly isolated organs (300 mg) were snap-frozen and kept at 80°C until use. Organs were homogenized in Qiazol lysis reagent using the Next Advance Bullet Blender (Next Advance, Inc. Troy, NY, USA). Homogenized samples were centrifuged at 18 800 g for 10 min to obtain clear lysates. All tissue processing was carried out at 4°C. Total RNA was extracted from the tissue lysates using the miRNeasy Mini Kit (Catalogue number 217004, Qiagen, Germantown, MD, USA) and quantified using the Nanodrop 8000 microvolume spectrophotometer (ThermoFisher Scientific). Real-time PCR reactions were set-up with 50 ng of RNA in duplicates. Genes of interest were evaluated using the TaqMan® Gene expression assay and the TaqMan® RNA-to-Ct™ 1-Step Kit (both from ThermoFisher) according to
the manufacturer’s instructions. Relative quantification was calculated with the standard ΔΔCt method; amplification signals from target gene transcripts were normalized to those from beta-glucuronidase (Gusb) transcripts. Relative fold induction was calculated by further normalization to gene transcripts from vehicle-treated animals. Gusb gene expressions were similar across all mouse strains used and across all organs within a given mouse strain (Figure S1A–C). Gusb gene expression in organs from control mice was similar to that from the corresponding organs from haemin-injected mice.

Haem quantification

Mice were perfused with PBS under anaesthesia. Harvested organs (300 mg) were homogenized in radio-immunoprecipitate assay buffer using the Next Advance Bullet Blender (Next Advance, Inc.). Homogenized samples were centrifuged at 18 800 g for 10 min to obtain clear lysates. All tissue processing was carried out at 4°C. Total haem in tissue lysates was quantified using a colourimetric assay kit (QuantiChrom haem assay kit; Bioassay Systems, Hayward, CA, USA) as described in previous studies (Ghosh et al., 2018). The bicinchoninic acid (BCA) assay kit (ThermoFisher Scientific, Catalogue number 23225) was used to quantify total protein in the lysates. Total haem values were first normalized to total protein, and then the haem values from vehicle-injected control organs were subtracted from the corresponding haem-injected organ values to determine the increase in haem in each organ after haemin injection.

HMOX1 and PGF protein quantification

Heart HMOX1 and PGF concentration were measured using the mouse HMOX1 enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY, USA) and the PGF ELISA kit (Sigma-Aldrich) following the manufacturer’s instructions. Total protein was quantified using the BCA method.

Statistical analysis

GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analyses. Results are reported as mean ± standard error of the mean. Group means were compared using parametric tests, such as t-test (for two groups) and one-way analysis of variance for more than two conditions. Statistical significance was set at P values of <0.05.

Study approval

The Institutional Animal Care and Use Committee at the University of Pittsburgh approved all experimental procedures performed on mice (Protocol number 16099101).

Results

Prominent cardiac expression of Hmox1 and Pgf in sickle cell mice

We observed differential expression of Hmox1 transcripts in untreated SS mice with highest expression level in the spleen, liver and kidney (P < 0.0001, Fig 1A). We hypothesized that uptake of haem from haemolysis induces this heterogeneous expression. To investigate this hypothesis, we increased circulating haem in SS mice with intravenous injection of haemin. Haem injection produced a sharp increase in Hmox1 gene expression in all organs analysed after 3 h. Hmox1 transcripts significantly increased, as expected, by about 20-fold in the kidney, 8-fold in the liver, 6-fold in the spleen, 5-fold in the lung and 4-fold in the BM after haem injection compared to vehicle injected controls (Fig 1). Surprisingly, the heart showed 18-fold induction of Hmox1, implying an unexpected capacity of the heart to take up haem and induce its degradation pathway. The spleen showed little increase in Hmox1 expression following haem injection, probably because its basal expression was already quite high in the spleens of the untreated SS mice (Fig 1A), presumably due to the high rate of splenic macrophage turnover of sickle erythrocytes. All the organs of AA strain control mice were similarly affected by haem injections. A second surprise was that the haemin-induced liver Hmox1 transcript level was significantly lower in SS mice than in AA mice (Fig 1C). This result implies that SS mice may have an impaired reserve capacity for induction of Hmox1, a vital enzyme for detoxifying and clearing haem.

Previous work from our laboratory (Sundaram et al, 2010; Wang et al, 2014) and others (Heeschen et al, 2003; Perelman et al, 2003; Peiskerova et al, 2013; Matsui et al, 2015; Gu et al, 2018; Kelaidi et al, 2018) has shown a link between haem, elevated PGF and vasculopathy in diseases with chronic haemolytic anaemia, such as SCD and thalassaemia. Therefore, we evaluated the expression of Pgf in the organs of SS mice, and determined that there was heterogeneity (P < 0.0001, Fig 1E) in basal Pgf expression in these mice, surprisingly with the highest expression in the heart (DeltaCt 2·9 ± 0·3), lung (3·1 ± 0·2) and kidney (5·0 ± 0·6). With haemin injection, cardiac Pgf transcript showed a robust 4-fold further increase compared to vehicle-treated control mice (Fig 1F, G). Cardiac expressions of PGF and HMOX1 in SS mice are surprisingly high, and induced to even higher levels by circulating haem.

Strong cardiac inducibility of Hmox1 and Pgf in haemin-treated wild type mice

We hypothesized that the haemin treatment of mice would be sufficient to replicate the organ-specific variability in Hmox1 and Pgf expression seen in SS mice. Nrf2+/+ mice were injected intravenously with vehicle or a higher dose of haemin (120 µmol/kg) and organs were harvested 3 h later. Hmox1 mRNA levels were significantly increased (P < 0·0001, Fig 2A)
in all organs from Nrf2+/+ mice that received haemin compared with vehicle controls. Most importantly, different organs demonstrated variable degrees of Hmox1 inducibility with an increase of 207-fold in the liver, 201-fold in the heart, 101-fold in the kidney, 69-fold in the lung and 7-fold in the spleen (Fig 2B). The strong inducibility of Hmox1 expression by haem in liver and kidney was expected, but comparable level of haem inducibility of Hmox1 in the heart is a novel observation. For Pgf expression, we observed the highest basal expression in the heart, followed by lung and kidney (P < 0.0001, Fig 2C). Following haem exposure, heart tissue had the highest elevation of Pgf expression (Fig 2C). Interestingly, haemin treatment of Nrf2+/+ mice largely recreated the organ-specific expression pattern of Pgf in untreated SS mice (Fig 1E compared to Fig 2C). This suggests that in sickle cell mice, haem exposure from haemolysis is a factor regulating organ-specific Pgf expression (Fig 2C). The high basal and haem-induced expression of Hmox1 and Pgf in the heart is remarkable.

**Organ-specific haem induction of Hmox1 is largely Nrf2 dependent**

The Nrf2 gene regulates the basal and inducible expression of Hmox1 and to understand the mechanisms and molecular pathways involved in the haemin-induced organ-specific Hmox1 expression, we utilized Nrf2 null mice. Similar to results from other strains described above, we observed organ-specific heterogeneity in haemin-induced Hmox1 expression in Nrf2−/− mice (Fig 2B), with the kidney (86-fold), heart (49-fold) and liver (44-fold) showing relatively higher induction of Hmox1. Most importantly, haemin induction of Hmox1 was largely Nrf2-dependent, with 59–78% lower response in Nrf2−/− mice in every organ except kidney. The 101-fold haem-induction of Hmox1 in the kidney was essentially Nrf2-dependent (Fig 2B). Interestingly, Pgf expression in the organs of Nrf2−/− mice (Figure S2) were similar to those of Nrf2+/+ mice (Fig 2C), with the basal expression of Pgf highest in the heart (2.15 DeltaCt), followed by lung and kidney (Figure S2). As observed in Nrf2+/+ mice, heart and lung had the highest expression of Pgf post-haemin exposure in Nrf2−/− mice. Haemin injections resulted in significant Pgf induction in both Nrf2+/+ and Nrf2−/− mice (Figure S3) with no significant change between the two strains, suggesting that haem-induced Pgf expression in the organs examined was Nrf2-independent.

**HMOX1 and PGF proteins are highly inducible by haem and Nrf2-dependent**

We confirmed at the protein level the strong cardiac inducibility of HMOX1 and PGF expression in the heart
tissue lysates 3 h after haem injection. We further analysed HMOX1 protein as a percentage of total heart protein in both vehicle and haemin treated animals. In agreement with our mRNA measurements above, HMOX1 protein increased by approximately 49% (P < 0.005), 57% (P < 0.001), and 33% (P < 0.05) in SS, Nrf2+/+ and Nrf2−/− mice respectively (Fig 3A). HMOX1 protein expression in the heart lysates was Nrf2-dependent in both vehicle-treated (P < 0.001) and haemin-injected mice (P < 0.05, Fig 3A), also supporting the mRNA measurements described above. In the same heart lysates, PGF protein as a percentage of total heart protein increased by about 22% (P < 0.01), 29% (P < 0.05), and 18% (P < 0.01) in SS, Nrf2+/+ and Nrf2−/− mice respectively (Fig 3B). However, the percentage of cardiac PGF was not significantly different between Nrf2+/+ and Nrf2−/− mice (Fig 3B).

Haem levels in different organs after haem injection correlates with Hmox1 induction

Our previous results consistently demonstrated a heterogeneous Hmox1 response to haemin among different organs that were examined, suggesting variability in haem degradation or haem distribution. To investigate this further, we measured intracellular haem in Nrf2+/+ and Nrf2−/− mice that received vehicle or haemin. The organs were perfused to remove circulating red blood cells before processing for haem quantification. There was variability in haem biodistribution 3 h after intravenous injection of haem (Fig 4A). Our results indicate that haem levels at 3 h remained significantly increased by about 67% (P < 0.05) in the spleen and 53% (P < 0.05) in the liver of Nrf2+/+ animals while the other organs were largely unaffected. Showing a remarkable degree of correlation, the organs with the highest haem induction.
of Hmox1 mRNA showed the lowest levels of intracellular haem, suggesting a more rapid clearance of haem by enhanced Hmox1 expression in the heart, liver, kidney and lung (Pearson $R^2 = 0.82$, $P = 0.03$, Fig 4B).

Nrf2 deficiency altered the haem distribution in haemin-injected mice. In haemin-injected Nrf2$^{−/−}$ animals, organ-specific haem levels were significantly higher by about 47% ($P < 0.05$) in the spleen, 58% ($P < 0.01$) in the kidney and 68% ($P < 0.05$) in the liver compared to haemin-injected Nrf2$^{+/+}$ mice. Interestingly, vehicle-treated Nrf2$^{−/−}$ mice showed mild but statistically significant haem accumulation in the heart ($P < 0.01$) and lung ($P < 0.05$) compared to Nrf2$^{+/+}$ mice, presumably due to basal state effects of Nrf2 deficiency on chronic metabolism of endogenous haem. After haemin injection, the Nrf2$^{−/−}$ mice accumulated significantly higher levels of haem in the heart ($P < 0.01$) and kidney ($P < 0.05$) compared to haemin-injected Nrf2$^{+/+}$ mice. This may be due to impaired inducible haem clearance in the heart and kidney of the Nrf2-deficient mice compared to Nrf2$^{+/+}$ mice (Fig 2B). The liver and lung haem concentrations were not significantly different between the Nrf2$^{−/−}$ and Nrf2$^{+/+}$ mice.

**Haem clearance is associated with haem transporter expression**

Our results above suggested that extracellular haem induced the expression of Hmox1 and Pgf genes in an organ-specific manner. Therefore, we hypothesized that differences in haem trafficking might contribute to this phenotype. Because Hrg1 functions as a haem transporter (Rajagopal et al, 2008; White et al, 2013), we evaluated Hrg1 mRNA expression in these organs. Haem injection significantly induces the expression of Hrg1 gene in most organs ($P < 0.05$), which was strongly dependent on Nrf2 in most organs (Fig 5A). Depending on the organ, Hrg1 induction by haem in was between 54% and 75% lower in Nrf2$^{−/−}$ mice. Compared to AA controls, SS mice showed significantly higher ($P < 0.05$) haem induction of Hrg1 in the spleen (~3-fold), liver (~3-fold) and kidney (~2-fold) (Fig 5B). Hrg1 induction closely paralleled the same organ-specific induction pattern as Hmox1 in relation to haem content, probably reflecting similar regulatory mechanisms ($R^2 = 0.82$, $P = 0.004$, Fig 5C).

**Discussion**

Sickle cell disease is no longer the ‘disease of childhood’ as described in the 1960’s (Maglione et al, 1993). Recent studies have shown that over 90% of the patients live to adulthood, especially in developed countries (Maglione et al, 1993; Platt et al, 1994). The improvement in survival is due to several interventions, such as new-born screening, use of penicillin prophylaxis and pneumococcal vaccination, hydroxyurea therapy and advancement in research and translational studies (Platt et al, 1994; Nouraie et al, 2013). These advances in medicine have contributed to better prognosis and quality of care for patients with SCD compared to five decades ago. Despite this progress in medicine and treatment of SCD patients, mortality in adult patients remains high (Platt et al, 1994). Organ failure due to accumulated injury from persistent haemolysis (Gardner et al, 2016) and sickling remains one of the major causes of death in patients with SCD (Vichinsky, 2017). The current study assessed organ-specific haem induction of Pgf and Hmox1, as a way to identify the most at-risk organs in SCD during elevation of circulating extracellular haem. The SCD pattern of Hmox1 and
Pgf expression is largely replicated by haem injection, mimicking acute haemolysis.

HMOX1 is a cytoprotective enzyme that metabolizes haem. Its deletion in mice amplified the severity of cerebral (Paulukonis et al., 2016) and noncerebral malaria (Seixas et al., 2009), ischaemia-reperfusion injury (Maitra et al., 2017), inflammation and vascular injury in different organs in SCD (Belcher et al., 2008), and ischaemia-reperfusion injury (Maitra et al., 2006; Belcher et al., 2010). Our data support and extend the prior work of Belcher et al. (2006, 2010), which showed Hmox1 expression and haem-inducibility in some organs of transgenic sickle mice. We have characterized the expression of Hmox1 in organs from naive and haemin-treated Townes sickle mice. We observed wide heterogeneity in the induction of transcript levels of Hmox1 in different organs, but, more importantly, we noticed a significant but very distinct haemin induction pattern depending on the organ. Spleen and liver showed the highest expression of Hmox1 as expected, as these are key organs in the reticuloendothelial system, involved in turnover of old and senescent red blood cells (RBCs). Exposure to extracellular haem further increased the transcript level of Hmox1 in these organs, with an unexpected strongest induction in the kidney and the heart of SS mice, which had lower baseline expression. The role of Hmox1 in the heart is controversial. Cardioprotective roles of Hmox1 in the heart includes reduction in oxidative stress (van Berlo et al., 2013), improved mitochondrial function (Hull et al., 2016), prevention of vascular remodelling (Mito et al., 2008), inhibition of apoptosis (Wang et al., 2010; Vichinsky, 2017) and prevention of atherosclerosis. Increased Hmox1 expression and activity is also linked to diabetes-induced oxidative stress in the heart via accumulation of iron (Farhangkhoe et al., 2003). Hmox1 expression in the heart of SS mice comprises part of the previously reported atheroprotective effect of SCD in SS/apolipoprotein E-deficient mice (Wang et al., 2013).

Interestingly, our result also suggests a previously unre- ported impaired inducible reserve capacity of Hmox1 induction in the liver (Fig 1C). Our finding is consistent with the published effectiveness of augmented Hmox1 expression as a protective therapy through gene transfer (Belcher et al., 2010) or through Nrf2 activators (Ghosh et al., 2016; Ghosh et al., 2018) in sickle cell mice. In agreement with the current literature (Boyle et al., 2011; Loboda et al., 2016), our data offers novel insights regarding the organ-specific Hmox1 expression in vivo. Although Nrf2 depletion ubiquitously blunted haemin-induced Hmox1 levels, the magnitude of the Hmox1 reduction varied in the different organs, suggesting the presence of additional unknown mechanisms that control Hmox1 expression. These mechanisms deserve future investigation.

Similar to Hmox1, we have investigated Pgf haemin-induc- tion in organs of SS, Nrf2+/+ and Nrf2−/− mice. For the first time, we describe high-level expression of Pgf in the heart of sickle cell mice. Pgf is expressed in several organs including placenta, erythroid cells, heart and skeletal muscle (Pamplona et al., 2007). We observed the highest Pgf expression in the heart, followed by the lung and kidney in organs isolated from naive adult SS mice. On exposure to extracellular haem in the circulation, we found a surprisingly high increase in Pgf transcript and total protein levels in the heart. Similarly, Pgf heart levels in haemin injected Nrf2−/− animals rose higher than all other organs. Elevated circulating level of Pgf protein is associated with worsening cardiac outcomes in patients with chronic kidney disease (Heeschen et al., 2003; Matsui et al., 2015), atherosclerosis (Cao et al., 1997) and asthma (Maglione et al., 1993). We have previously reported the association between Pgf, inflammation, pulmonary hypertension (PH)
and high mortality in patients with SCD (Perelman et al., 2003; Sundaram et al., 2010). This present result, showing the highest basal expression of Pgf in the heart followed by the lung in SS and Nrf2\textsuperscript{+/−} mice, is unexpected and in contrast to previous reports of low expression levels in these organs (Alam et al., 2003; Liu et al., 2005; Pamplona et al., 2007). This apparent inconsistency might be due to differences in mouse strains, although our own results were consistent across four different strains of mice. Additionally, our group and others have shown that erythroid hyperplasia, hypoxia and erythropoietin in SCD contribute to elevated circulating PGF levels in both humans and mice with SCD (Tordjman et al., 2001; Perelman et al., 2003; Sundaram et al., 2010; Wang et al., 2014). High expression of Pgf in the heart is associated with angiogenesis and ischaemia (Maglione et al., 1993), though its role in cardiovascular disorders is somewhat controversial. Kolakowski et al., (2006) reported that direct intramyocardial injection of PGF following a large myocardial infarction attenuated adverse ventricular remodelling and improved myocardial function. On the other hand, elevated serum PGF is an independent risk factor for mortality (Matsui et al., 2015) and left ventricular diastolic dysfunction in patients with chronic kidney disease (Peiskerova et al., 2013). PGF contributes to cardiac hypertrophy (Dewerchin & Carmeliet, 2012; Eiymo Mwa Mpollo et al., 2016, Roncal et al., 2010), through intermediate paracrine growth factors such as interleukin 6 (Accornero et al., 2011; Dewerchin & Carmeliet, 2012), which is known to be elevated in the serum of SCD patients (Taylor et al., 1995; Qari et al., 2012; Saray et al., 2015).

The third gene that we evaluated was the haem transporter Hrg1, which is known to facilitate haem export from the phagolysosome into the cytosol during RBC recycling in macrophages (Rajagopal et al., 2008; White et al., 2013). Our results describe, for the first time, how Hrg1 is regulated in vivo by the addition of an extracellular haem analogue with or without Nrf2. Similar to Hmox1 and Pgf, HRG1 expression in Nrf2\textsuperscript{+/−} mice was stimulated by haemin injections in an organ specific manner. Furthermore, the absence of Nrf2 in the Nrf2 null animals eradicated the haemin effect in all organs in agreement with a previous publication reporting the role of NRF2 in HRG1 regulation (Alam et al., 2003). Interestingly, Hrg1 induction by haemin strongly correlates with intracellular haem content in haemin-injected mice. Moreover, intracellular haem content strongly correlates with Hmox1 expression in the same mice. The relationship of haem transport to haem clearance requires further investigation.

It is interesting that haemin injection in wild-type mice largely recreates the profile of Hmox1 and Pgf expression by organ seen in untreated SS mice. This suggests that sickling, vaso-occlusion or anaemia is not specifically required for this Hmox1 and Pgf pattern. Further stimulation of the SS mice with additional haem simply amplifies this pattern, but retains the dominance of liver and heart expression of Hmox1 and Pgf. Although the change in expression of haem-regulated genes is not surprising in liver and spleen, organs involved in haem clearance, we report previously underappreciated Hmox1 basal and inducible expression in the heart.

We found prominent organ-specific Hmox1 and Pgf expression patterns, which could promote variable protective and maladaptive responses among these organs. A recent investigation of blockade of PGF suggested beneficial effects in SS mice (Gu et al., 2018). The remarkable regulation of Hmox1, Pgf and Hrg1 in the heart by haem offers a particular new avenue for investigation.

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**Author contributions**

OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript.

**Conflicts of interest**

The authors declare no competing financial interest in relation to the study.

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**Supporting Information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig S1.** Similarity in GUSB expression in vehicle and haem-injected mice.

**Fig S2.** Basal PlGF expression is highly variable in Nrf2\textsuperscript{+/−} mice.

**Fig S3.** Haem-induced PlGF expression in solid organs is not Nrf2 dependent.

**Table S1.** Rt-qPCR Taqman probes.


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