Glucocorticoids improve erythroid progenitor maintenance and dampen Trp53 response in a mouse model of Diamond–Blackfan anaemia

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Summary

Diamond–Blackfan anaemia (DBA) is a rare early-onset red cell aplasia that is usually manifested during the first year of life (Diamond & Blackfan, 1938; Nathan et al., 1978). At diagnosis, patients present macrocytic anaemia, reticulocytopenia and a normocellular bone marrow where erythroid progenitors are scarce or absent (Vlachos & Muir, 2010). The finding of mutations in ribosomal protein s19 (RPS19) led to the notion that DBA is a ribosomopathy causing functional haploinsufficiency (Draphtchinskaia et al., 1999; Farrar & Dahl, 2011). Mutations in RPS19 are found in 25% of all patients and, together with more than 10 other less frequently affected ribosomal protein genes, they cover 60–70% of all DBA cases (Gazda et al., 2004; Landowski et al., 2013; Vlachos et al., 2013). In 2012 the erythroid specific transcription factor GATA1 was found to be mutated in a small cohort of DBA patients, challenging the notion that DBA is a disease of exclusive ribosomal origin (Sankaran et al., 2012).

Diamond–Blackfan anaemia patients usually require lifelong treatment and apart from chronic blood transfusions, glucocorticoid therapy has been standard for more than half a century to alleviate the anaemia (Gasser, 1951; Vlachos et al., 2008). Up to 80% of all DBA patients respond to glucocorticoid therapy and approximately 40% remain steroid responsive over time (Vlachos et al., 2008). Despite being the only available pharmacological therapy for DBA, the therapeutic mechanisms behind glucocorticoid treatment remain elusive. It is well established that glucocorticoid receptor activation is essential for erythroid progenitor expansion during stress erythropoiesis that occurs upon...
excessive blood loss or chronic anaemia. Mice lacking the ability of glucocorticoid receptor dimerization fail to respond to stress erythropoiesis caused by haemolytic anaemia (Bauer et al., 1999) and self-renewing colony assay of erythroid progenitors ex vivo requires glucocorticoids (Wessely et al., 1997; Leberbauer et al., 2005; Dolznig et al., 2006; Flygare et al., 2011). Multiple glucocorticoid-induced genes are important for erythropoiesis, such as the RNA-binding protein Zfp36l2, which has proved crucial for self-renewal of erythroid progenitors (Zhang et al., 2013) and the transcription factor Myb, which is important for long-term erythroid proliferation (Wessely et al., 1997; Lindern von et al., 1999). Glucocorticoids also delayed differentiation in both human and avian erythroid cells, which in turn led to an increase in the expansion capacity of erythroid progenitors (Golde et al., 1976; Wessely et al., 1997; Lindern von et al., 1999). Thus, glucocorticoids play an important role in the stressed erythroid system and are important in DBA treatment, but less is known about their therapeutic mechanisms. In order to elucidate this, more knowledge about disease mechanisms in DBA is crucial.

Elevated TP53 activity is brought forward as one of the underlying mechanisms behind DBA pathology, and studies in human cells as well as in mice and zebrafish have shown improvement of the DBA phenotype in the absence of Trp53 (Danilova et al., 2008; Dutt et al., 2011; Jaako et al., 2011; Taylor et al., 2012). In the context of intact ribosomal proteins, erythroid cells with a Trp53 -/- background proliferate better than wild type counterparts, suggesting that Trp53 has a general inhibitory effect on erythropoiesis (Ganguli et al., 2002). It is not fully known how Trp53 is activated in ribosome protein-deficient erythroid cells, but it is probably explained in part by stress responses due to impaired ribosomal biogenesis, known as ribosomal stress.

The aim of this study was to identify the therapeutic mechanisms of glucocorticoids in DBA by validating the response in an Rps19-deficient mouse model and verifying these results in DBA patient cells. Our mouse model proved to be an excellent tool to validate DBA drug treatment and responded to glucocorticoids similarly to DBA patients. We found that glucocorticoid treatment prevented erythroid progenitor depletion and delayed erythroid differentiation, which led to improved expansion of Rps19-deficient cells. The increased Trp53 activity in Rps19-deficient cells was reversed by glucocorticoid treatment, indicating a disease specific mechanism of glucocorticoids.

Material and methods

Rps19-deficient mouse strain, transplantation and shRNA induction

We used a doxycycline-inducible Rps19-deficient mouse previously developed by us, carrying a reverse transactivator (rtTA)-dependent shRNA against Rps19 that develops robust anaemia upon doxycycline induction. For all experiments, we used the shRNA-D mice (Jaako et al., 2011). The haematopoietic phenotype in these mice is specific to Rps19 down regulation as it can be cured by enforced expression of Rps19 (Jaako et al., 2014). 8- to 14-week-old WT C57bl/6 males were subjected to lethal irradiation (900 cGy) and transplanted with unfractionated bone marrow from Rps19+/+, rtTA/rtTA (carrying transactivator only) or D/+, rtTA/rtTA recipients (carrying transactivator and heterozygous for Rps19 shRNA). The animals received Ciprofloxacin 125 µg/ml (Arrow Generics, Stevenage, UK) for 2 weeks post-transplantation. Six to nine weeks after transplantation the mice received doxycycline (2 mg/ml) with sucrose (10 mg/ml) in drinking water ad libitum for 9 days as well as 100 µg/ml of Prednisolone or corresponding volume of solvent in the drinking water (Sigma-Aldrich, St. Louis, MO, USA). Peripheral blood was analysed fresh on a haematology analyser KY-21N (Sysmex, Kobe, Japan) after 9 days of doxycycline induction.

Liquid culture and methylcellulose colony assay

Faetal livers from heterozygote D/+ E14.5-15.5 embryos were dissected and enriched for KIT (CD117) positive cells using magnetic beads and LS columns (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturers’ instructions. The cells were kept in serum-free expansion medium (SFM; Stem Cell Technologies, Vancouver, Canada) with 2 µ/ml erythropoietin (EPO; LEO Pharma, Ballerup, Denmark), 100 ng/ml murine stem cell factor (mSCF) (ProTech, Rocky Hill, NJ, USA), with or without 100 nmol/l Dexamethasone (Sigma-Aldrich), and/or 0.5-1 µg/ml doxycycline (Sigma-Aldrich) and kept at 37°C with 5% CO2. Cells were counted manually using trypan blue in a haemocytometer. Colony forming unit-erythroid (CFU-E) assays were performed using serum free semisolid MethoCult #3236 (Stem Cell Technologies) supplemented with 5 u/ml EPO and 50 ng/ml mSCF and were cultured at 37°C with 5% CO2 and 4%O2, and the colonies were scored after 4 days.

Real time-polymerase chain reaction (RT-PCR)

For real time (RT)-PCR analysis, RNA extraction was performed using RNeasy micro kit (Qiagen, Venlo, Netherlands) according to the manufacturers’ instructions and analysis was performed on a 7900 HT fast RT-PCR (Applied biosystems, Waltham, MA). Primers used for RT-PCR were from Life Technologies (Carlsbad, CA, USA): Hprt (#0154399_m1), GAPDH (#02758991_g1), Cdkn1a (#04205640_g1 and 00355782_m1), Bax (#00432051_m1 and 0180269_m1), Congl (#00438084_m1), Phlda3 (#00449846_m1), Ptp4a3 (#00477233_m1) and ZFP36L2 (#00272828_m1), and Zmat3 (#01292424_m1).

Flow cytometry

A modified version of an erythroid differentiation protocol was used for flow cytometric analysis (Liu et al., 2013). Antibodies used were phycoerythrin-cyanin 7 (PECy7)-conjugated
TER119 (eBioscience, San Diego, CA, USA), and phycoerythrin (PE)-conjugated CD44 (BioLegend, San Diego, CA, USA). Allophycocyanin (APC)-conjugated B220, CD3 and GR-1 (BioLegend) were used as lineage markers. 7-aminoactinomycin D (7AAD) was used to monitor viability. The plots were gated on live cells of appropriate size, negative for lineage markers. For flow cytometric detection of apoptosis, PE-conjugated AnnexinV apoptosis detection kit (BD biosciences, Franklin Lakes, NJ, USA) was used according to the manufacturer’s instructions. Data collection was done on a Canto II flow cytometer (BD Biosciences). For cell cycle analysis, the cells were disrupted in ice-cold ethanol and the DNA was stained with propidium iodide. Cell cycle analysis was done on Aria III flow cytometer (BD Biosciences). All analyses were performed using FlowJo v10 (Tree Star Inc., San Carlos, CA, USA).

**Western blot assay**

Cells cultured for 72 h were harvested in Laemmli buffer containing 1% mercaptoethanol and proteinase inhibitor cocktail, homogenized and boiled for 5 min. Samples were run on a gradient 4–12% Bis-Tris gel (Life Technologies), and blotted according to manufacturer’s instructions. The anti-MYB antibody was from Santa Cruz Biotechnology (Dallas, TX, USA) and anti-Actin antibody from BD Biosciences.

**DBA patient cells**

A human peripheral blood sample was collected after obtaining informed consent according to ethical guidelines at Universitätsklinikum in Freiburg, Germany. The patient sample was from a 14-year-old transfusion-dependent male DBA patient carrying a heterozygous RPS19 mutation (c.280C>T, p.Arg94X), and healthy control samples were from one male and one female. The blood was enriched for CD34+ fraction using magnetic MicroBead Kit, according to manufacturer’s instructions. Data collection was done on a Canto II flow cytometer (BD Biosciences). All analyses were performed using FlowJo v10 (Tree Star Inc., San Carlos, CA, USA).

**Statistical analysis**

For statistical analyses the nonparametric unpaired Mann–Whitney U test or paired Wilcoxon test were employed, unless otherwise stated. All statistical calculations were done in the software GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). All graphs display median with interquartile range, unless otherwise stated. P-values: *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001.

**Microarray statistics**

The microarray data were Robust Multiarray Average (RMA) normalized. Differentially expressed genes were identified using Significance Analysis of Microarrays (SAM), using a ‘multi class’ analysis, where a value of delta was chosen so the resulting differentially expressed genes had a false discovery rate (FDR) of 5%. The differentially expressed genes were then clustered into nine partitions using k-means. Gene set enrichment analysis (GSEA) was performed using gene sets defined by the following: Trp53 genes were taken from KEGG (http://www.genome.jp/kegg/). TER119 and burst forming unit-erythroid (BFU-E) genes were defined from RNA sequencing data http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26086 (Flygare et al, 2011). M/A values were calculated where genes with A > 3 were selected first. Of the remaining, the top and bottom 330 genes were taken from a ranked list of M (log ratios) values to define each list. GSEA was then performed using default settings.

**Results**

**Glucocorticoids reverse anaemia in a mouse model of DBA**

In our Rps19-deficient inducible mouse model 9 days of doxycycline administration caused a robust anaemia with decreased red blood cell (RBC) counts, reduced haematocrit (HCT) and haemoglobin (HGB) levels, as previously reported (Fig 1A–C) (Jaako et al, 2011). The anaemia of Rps19-deficient mice (D/) was significantly improved when treated with the glucocorticoid Prednisolone, with a significant increase in RBC counts as well as HGB and HCT compared to untreated Rps19-deficient animals. Rps19-deficient mice that received Prednisolone also showed significantly lower MCV compared to untreated Rps19-deficient mice (Fig 1D). As previously reported, Rps19-deficiency led to a decrease in white blood cell counts (WBC) and platelet (PLT) counts (Jaako et al, 2011), which were further decreased in prednisolone-treated animals (Fig S1). These results demonstrate that our Rps19-deficient mouse model recapitulates the anaemia seen in DBA, and that these mice respond to glucocorticoid treatment similar to DBA patients with improved erythroid blood parameters.

**Glucocorticoids improve survival and expansion of Rps19-deficient erythroid cells**

Erythroid cells from patients and DBA cell lines have shown to be pro-apoptotic, which indicates that apoptosis
plays an important role in DBA pathogenesis (Perdahl et al., 1994; Miyake et al., 2008). Therefore, we examined apoptosis in Rps19-deficient erythroid cells when treated with the synthetic glucocorticoid Dexamethasone. As expected, we observed that Rps19-deficient erythroid cells displayed elevated levels of apoptosis in vitro, shown by an increase in the AnnexinV+/7AAD fraction. Dexamethasone treatment significantly decreased the apoptotic fraction of Rps19-deficient cells (Fig 2A), and it also reduced apoptotic levels in healthy cells. This indicates that glucocorticoids have a pro-survival effect on erythroid cells in general, and on Rps19-deficient cells in particular. In addition to being more pro-apoptotic, erythroid progenitors from both DBA patients and an Rps19-deficient mouse model failed to expand in liquid culture (Hamaguchi et al., 2003; Miyake et al., 2005; Jaako et al., 2011). To examine the effect of glucocorticoids on the expansion capacity of Rps19-deficient cells, erythroid expansion cultures were maintained for 7 days. The expansion of Rps19-deficient erythroid progenitors was dramatically decreased compared to controls as early as 48 h after doxycycline administration, and did not recover during the 7-day culture period (Fig 2B). Rps19-deficient cells receiving Dexamethasone significantly increased their expansion and allowed the culture to be maintained for the full 7 days. As seen before, healthy control cells expanded better in the presence of Dexamethasone, indicating that glucocorticoids promote expansion of erythroid cells in general. Together, these results show that glucocorticoid treatment decreases apoptosis and promotes expansion of Rps19-deficient erythroid cells and that these effects are more pronounced in Rps19-deficient cells than in healthy cells.

Glucocorticoids counteract erythroid differentiation and erythroid progenitor depletion caused by Rps19-deficiency

Glucocorticoids improve the phenotype of Rps19-deficiency both in vivo and in vitro and we therefore wanted to identify changes in erythroid gene expression caused by glucocorticoid treatment. We performed a microarray analysis on KIT-enriched fetal liver cells cultured for 24 h in the following conditions: Healthy cells with and without Dexametha-
sone and Rps19-deficient cells with and without Dexamethasone. KIT-enriched fetal liver cell population mostly contains burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E) progenitors, as well as some immature erythroblasts not yet expressing TER119. Genes significantly altered by either Rps19-deficiency or Dexamethasone treatment were mapped against previously published RNA sequencing data of healthy cells from BFU-E or TER119+ cells from murine fetal liver (Flygare et al, 2011). In this previously published data set, the BFU-E compartment consisted of fetal liver cells highly purified for the early erythroid progenitor BFU-E by cell sorting. The fraction sorted on positive TER119 expression was composed of more differentiated erythroblasts. In both these fractions, the 330 most highly expressed genes were selected for GSEA (Fig S2A). Genes up regulated by Dexamethasone treatment of healthy cells were much more associated with BFU-Es than with TER119+ cells (FDR q = 0.032), indicating that glucocorticoids promote maintenance of erythroid progenitors (Fig 3A). Several genes were up regulated by Rps19-deficiency and these were much more associated with TER119+ cells (FDR q = 0.011) than with BFU-Es. This was an indication that Rps19-deficiency caused increased erythroid differentiation by up regulation of genes associated with the more mature erythroblast TER119+ fraction (Fig 3B). Comparison of Rps19-deficient cells with or without dexamethasone treatment showed that glucocorticoid treatment caused up regulation of genes highly associated with BFU-Es (FDR q = 0.062) (Fig 3C). Together, this demonstrates that glucocorticoids promote maintenance of erythroid progenitors and delay the increased differentiation and depletion of erythroid progenitors seen in untreated Rps19-deficient cells. To further identify dexamethasone-inducible genes that could be responsible for the delayed differentiation and decrease in erythroid progenitor maintenance we compared samples from the microarray analysis cultured under the following three conditions: (i) Rps19-deficient cells, (ii) Rps19-deficient cells + dexamethasone and (iii) Wild type controls not receiving dexamethasone. Gene clustering was performed on differentially expressed genes in these conditions. We identified two sets of differentially expressed genes that were normalized by dexamethasone (Cluster 2 and 7 in Fig S2B). Among the genes down regulated by Rps19-deficiency but reversed by glucocorticoids, we found Kit and Myc, which both are more highly expressed in erythroid progenitors than mature erythroid cells (Suppl Table I). Another transcription factor important for erythropoiesis induced by glucocorticoids is Myb, which is most highly expressed in erythroid progenitors but down regulated with differentiation (Emilia et al, 1986). We observed a significant decrease of Myb gene expression as well as MYB protein levels in Rps19-deficient cells observed previously (Sieff et al, 2010). At the gene expression level, glucocorticoid treatment significantly increased Myb expression to normal levels (Fig S3). Together, these results indicate that erythroid differentiation is increased in Rps19-deficient cells and that glucocorticoid treatment counteracts this by maintaining the cells in a more immature erythroid progenitor state. These effects were observed as early as after 24 h of glucocorticoid treatment.

**Glucocorticoids maintain erythroid progenitors and delay differentiation of Rps19-deficient cells**

During the course of in vitro erythroid differentiation in liquid culture there is a progressive loss of cells with early erythroid progenitor potential, such as CFU-E cells, and an accumulation of more mature erythroid cells expressing TER119. Our gene expression analysis indicated that glucocorticoid treatment of Rps19-deficient cells maintained erythroid progenitors and delayed erythroid differentiation (Fig 3). To validate these findings, we examined CFU-E colony forming potential of KIT-enriched Rps19-deficient and healthy fetal liver cells in liquid culture. We hypothesized that CFU-E potential would decrease in Rps19-deficient cultures compared to controls over time, and that glucocorticoid treatment could rescue this depletion. As expected, Rps19-deficient cells showed decreased CFU-E colony forming potential, starting as early as 24 h after induction with doxycycline (Fig 4A). Dexamethasone treatment significantly retained cells with CFU-E potential in both Rps19-deficient and healthy cultures after 72 h. This trend was observed as early as 24 h of culture. CFU-E potential for glucocorticoid treated Rps19-deficient cells was equal to that of healthy controls, indicating that glucocorticoids can reverse erythroid progenitor depletion significantly (Fig 4A).

To further examine the maintenance of early erythroid progenitors and identify changes in differentiation between Rps19-deficient cells and controls, we employed flow cytometric analysis of the well-established markers of erythroid differentiation CD44 and TER119 (Fig 4B) (Liu et al, 2013). Rps19-deficient erythroid cells showed increased erythroid differentiation compared to controls, with less cells in the immature CD44+TER119+ fraction and more cells in the differentiated CD44+TER119- fraction after 48 h of culture. Dexamethasone treatment significantly reversed this and retained more cells in the immature CD44+TER119- fraction (Fig 4C). These findings were in line with data from the microarray analysis, indicating that increased differentiation of Rps19-deficient cells and depletion of erythroid progenitors could be reversed by glucocorticoid treatment.

During differentiation, erythroid cells undergo a set of cell divisions before they become mature red cells and exit the cell cycle. Cell cycle analysis of Rps19-deficient cells revealed that these cells to a greater extent reside in the G1 phase and less in S and M phase compared to healthy controls (Fig 4D). This cell cycle difference was not significantly reversed by glucocorticoid treatment. Together, these results demonstrate that Rps19-deficiency increases erythroid differentiation and depletes erythroid progenitors. Importantly, glucocorticoid administration significantly reverses both these
Fig 3. Rps19-deficiency increases erythroid differentiation and this is counteracted by dexamethasone (Dex). GSEA was performed on differentially expressed genes from microarray analysis mapped against genes most highly expressed in BFU-Es and TER119+ cells from fetal liver (Flygare et al., 2011). Enrichment of genes associated with BFU-E or TER119+ erythroid fractions between (A) Control and control + dexamethasone samples, (B) Control and Rps19-deficient samples, (C) Rps19-deficient and Rps19-deficient + dexamethasone samples.
Fig 4. Glucocorticoids prevent erythroid progenitor loss and decrease differentiation in Rps19-deficiency. (A) Healthy and Rps19-deficient KIT-enriched fetal liver cells cultured with and without dexamethasone for 24, 48 or 72 h and then plated for colony assay, in three separate experiments. (B) Flow cytometric analysis of the erythroid differentiation markers CD44 and TER119 in Rps19-deficient and healthy cultures with or without Dexamethasone treatment. Shown here are representative plots for each condition and time point from three separate experiments. (C) Quantification of data from the flow cytometric experiment in (B). (D) Cell cycle distribution analysis of cells cultured for 72 h. Displayed here is mean with standard deviation and paired student’s t-test was used for statistical analysis.
effects, which allows expansion of erythroid progenitors, despite Rps19-deficiency.

**Glucocorticoids reverse up regulation of Trp53 targets caused by Rps19-deficiency**

The tumour suppressor Trp53 is known to be highly involved in the DBA phenotype, as shown in animal models of DBA (Danilova et al, 2008; Taylor et al, 2012). In our mouse model, Rps19-deficient erythroid cells demonstrated a dramatic up regulation of Trp53 downstream target genes, including Cdkn1a (p21) and Bax that regulate cell cycle and apoptosis (Jaako et al, 2011). Activation of Trp53 in the Rps19-deficient mice involves 5S ribonucleoprotein particle (5S RNP)-mediated inhibition of MDM2. Deletion of the Trp53 gene or disruption of the 5S RNP-MDM2 interaction in the Rps19-deficient DBA mouse ameliorates the anaemia in vivo (Jaako et al, 2015). We therefore wanted to examine the effect of glucocorticoid treatment on Trp53 activity in Rps19-deficient erythroid cells. The expression of multiple Trp53 target genes was significantly elevated in Rps19-deficient erythroid cells, shown by GSEA of microarray data mapped against the Trp53 KEGG pathway (Fig 5A). The same thing was seen for multiple Trp53 target genes including Cdkn1a and Bax by RT-PCR (Fig 5B–C). Dexamethasone treatment significantly blocked the up regulation of multiple Trp53 target genes, demonstrating that glucocorticoids have a potent dampening effect on Trp53 activity in Rps19-deficient cells (Fig 5C). Glucocorticoids clearly displayed this effect after 72 h, but a trend was also seen after 24 h of treatment (compare Fig 5B and 5C). Importantly, glucocorticoids did not down regulate expression of the Trp53 target genes in healthy cells, indicating that glucocorticoids had this effect specifically in cells with elevated Trp53 activity. Both Rps19-deficient and control cells responded to glucocorticoid stimulation by up regulation of the glucocorticoid receptor target gene Perl at both 24 and 72 h (Fig 5A–B).

In order to determine if glucocorticoid receptor activation reduces nuclear TP53 levels we performed a mini screen with selected steroid-associated compounds including 30 glucocorticoid receptor agonists. The readout of the screen was average nuclear TP53 staining intensity in RPS19-deficient human A549 (TP53 wild-type) cells (Fig 5S). While RPS19-deficiency caused a dramatic increase in nuclear TP53 protein levels, this increase was moderately but significantly reduced when cells were treated with the group of glucocorticoid compounds. Neither androgens nor oestrogens had this effect. The results suggest that glucocorticoids are able to reduce nuclear TP53 protein accumulation caused by ribosomal stress.

To validate the clinical relevance of this effect, we examined gene expression of Cdkn1a in erythroid cells from one DBA patient carrying an RPS19 mutation. Indeed, the expression of CDKN1A was highly elevated in this patient compared to healthy donors, and CDKN1A expression was dampened after only 24 h of glucocorticoid treatment (Fig 5D). The patient cells were responsive to glucocorticoids, shown by up regulation of ZFP36L2, a direct target of the glucocorticoid receptor important for erythroid progenitor self-renewal (Fig 5C). Overall, these results demonstrate that glucocorticoids counteract up regulation of multiple Trp53 target genes in Rps19-deficient erythroid cells. This effect is not seen in healthy controls, which indicates that it is specific to DBA pathology.

**Discussion**

Glucocorticoids are often chosen as first line treatment for DBA, and they have now been used to treat DBA patients for more than half a century (Gasser, 1951). It is well known that glucocorticoids are crucial for stress erythropoiesis and self-renewal of erythroid progenitors in vitro, but little is known about their therapeutic mechanisms in DBA treatment. One previous attempt has been made to study glucocorticoid responsiveness in a mouse model of Rps6-deficiency, but these mice did not respond to glucocorticoid treatment (Keel et al, 2012). Here, for the first time, we show glucocorticoid responsiveness in a mouse model of DBA, demonstrating that it is a relevant and useful model to study therapeutic mechanisms of DBA treatment. We also demonstrate that glucocorticoids prevent the depletion of erythroid progenitors and the increase in erythroid differentiation observed in Rps19-deficient cells. Furthermore, we demonstrate that glucocorticoids inhibit up regulation of multiple Trp53 target genes, an effect not seen in healthy cells and thus specific to the DBA phenotype.

Erythropoiesis requires a tight regulation between self-renewal and differentiation of erythroid progenitors to generate sufficient numbers of mature red blood cells. Steady state erythropoiesis involves a set number of differentiation divisions and is regulated by EPO and SCF (Muta et al, 1995). Glucocorticoids are not required for steady state erythropoiesis, but are vital for situations of stress erythropoiesis, such as excessive bleeding or severe anaemia. This is best illustrated by lack of recovery from haemolytic anaemia of mice with disrupted glucocorticoid receptor function (Bauer et al, 1999). In steady state erythropoiesis, cultures treated with glucocorticoids give rise to more BFU-E colonies than untreated controls, indicating that they are important for erythroid progenitor self-renewal and maintenance (Flygare et al, 2011; Narla et al, 2011). We observe that Rps19-deficient erythroid cells have decreased capacity of CFU-E formation, which leads to decreased erythroid output and thus a decreased number of cells able to reach erythroid maturation. This is in line with other studies showing decreased CFU-E and BFU-E colony-forming abilities in erythroid cells from DBA patients (Nathan et al, 1978; Lipton et al, 1986; Giri et al, 2000). We show that glucocorticoid-treated Rps19-deficient cultures retain more CFU-E colony-forming capacity compared to their untreated counterparts, indicating that
Glucocorticoid treatment can significantly rescue erythroid progenitor depletion. This is in line with a study observing an increase in the erythroid progenitor population of steroid responsive DBA patients compared to transfusion-dependent patients (Iskander et al., 2015). In human bone marrow the erythroid output was decreased when RPS19 was knocked down and, similar to our findings, this was rescued by glucocorticoids (Ebert et al., 2005).

In our study, gene expression analysis demonstrated a signature of increased differentiation of Rps19-deficient cells.

Fig 5. Trp53 target genes are up regulated in Rps19-deficient cells, but not in the presence glucocorticoids. (A) GSEA of genes significantly up regulated by Rps19-deficiency mapped to the Trp53 KEGG pathway. (B) Trp53 responsive gene expression after dexamethasone and doxycycline treatment for 24 h and (C) 72 h. Results from three separate experiments. (D) Gene expression of CDKN1A in blood cells from peripheral blood of one DBA patient carrying an RPS19 mutation and two healthy control subjects.
The gene expression results further suggested that glucocorticoid treatment of Rps19-deficient cells decreased differentiation and increased maintenance of erythroid progenitors. This explanation to the therapeutic effect of glucocorticoids was confirmed by colony forming, cell expansion and flow cytometry analyses, demonstrating that glucocorticoid treatment reversed both increase in differentiation and depletion of erythroid progenitors in Rps19-deficient cells. It could seem counterproductive for glucocorticoids to delay differentiation in an Rps19-deficient erythroid system, but we postulate that this, together with increased maintenance of erythroid progenitors, prevents the system from being prematurely differentiated, which would result in exhaustion of self-renewing cells. Counteracting this exhaustion could allow erythroid expansion to occur despite Rps19-deficiency. This would be in line with the significant improvement of anaemia we observed in our mouse model of DBA.

TP53 has been identified a key player in the pathogenesis of DBA in both fish and human model systems (Danilova et al., 2008; Dutt et al., 2011; Taylor et al., 2012). Similarly to others, we have also shown that the anaemia of our Rps19-deficient mouse model could be significantly improved in a Trp53 null background (Jaako et al., 2011). Likewise, the phenotype of a mouse model of the ribosomal disease Treacher Collins syndrome is reversed in a Trp53 null background, demonstrating an important role for Trp53 in the pathology of ribosomopathies (Jones et al., 2008). The mechanism behind Trp53 activation in DBA is not fully understood, but probably involves activation of ribosomal stress pathways. In situations where one ribosomal component is not available in sufficient amounts, ribosome biogenesis fails and the ribosomal proteins RPL5, RPL11 and 5S rRNA diffuse from the nucleolus and act as sensors of ribosomal stress by forming a complex that inhibits MDM2. As MDM2 is a E3 ubiquitin ligase that promotes degradation of TRP53, formation of this complex leads to TRP53 stabilization (Lohrum et al., 2003; Zhang et al., 2003; Dai & Lu, 2004; Donati et al., 2013). Some studies have reported that erythroid defects in mouse cells and zebrafish models of ribosomal deficiency are not always corrected by Trp53 depletion (Torihara et al., 2011; Jia et al., 2013; Singh et al., 2014). Although TP53 seems to be important for the disease, it is plausible that there is more to the pathology of DBA than increased TP53 activity alone. Interestingly, both RPL5 and RPL11 are found mutated in DBA, but in these cases it is
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Author contributions

SES and JF designed the research study, SES, KS and MW performed the research, SES analysed the data and performed statistical analyses, SS performed statistical analysis of the microarray data, MW provided DBA patient samples, AJG and RDH performed research on the human cell line, P) and SK gave intellectual input to the study and provided the mouse model, and SES and JF wrote the manuscript.

Competing interests

The authors have no competing interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and methods.

Fig S1. Blood parameters of Rps19-deficient and healthy mice.

Fig S2. Gene expression analyses of microarray and RNA sequencing data.

Fig S3. Decreased Myb expression is normalized by glucocorticoids in Rps19-deficient cells.

Fig S4. Murine and patient cells respond to glucocorticoid stimulation.

Fig S5. TP53 protein is less upregulated in RPS19-deficient human cells treated with glucocorticoids.

Table S1. List of genes significantly altered by Dexamethasone treatment of Rps19-deficient cells. Log2 gene expression values of genes from cluster 2 and 7 from Fig S2.

unclear if TP53 activation has a role in the pathogenic mechanism (Singh et al, 2014) and more studies are needed to elucidate if ribosomal stress and TP53 activation is a central pathogenic mechanism in DBA. In this study, we found both a significant up regulation of TP53 protein and multiple TP53 responsive genes in both human and murine Rps19-deficient cells. Also, our microarray analysis reveals a strong Trp53 activation signature in Rps19-deficient erythroid cells. Glucocorticoids reverse the increased expression of multiple Trp53 target genes involved in both cell cycle (Cdkn1a and Ccng1) and apoptosis (Bax). Interestingly, no change in Trp53 target gene expression was seen in healthy cells regardless of glucocorticoid administration, indicating that this mechanism is specific to Rps19-deficient cells and thus disease-specific for DBA. Neither treatment with androgen- nor oestrogen-associated compounds resulted in any decrease of TP53 protein levels, indicating that this effect is specific to glucocorticoids. This effect was seen with treatment of multiple glucocorticoids, leading us to conclude that glucocorticoid receptor activation can have a TP53 dampening effect. One previous study reported negative cross talk between TP53 and the glucocorticoid receptor in a neuroblastoma cell line (Sengupta et al, 2000), but because glucocorticoids have very cell type-specific effects, it remained highly unclear if this was also true in a DBA setting. Here, we show, for the first time, that glucocorticoids dampen the elevated Trp53 activity seen in DBA, in a disease-specific manner.

Together, the results from this study demonstrate that glucocorticoid treatment dampens the elevated Trp53 activity seen in erythroid cells from DBA patients and murine cells, by decreasing the expression of multiple Trp53 responsive genes, coupled with decreased levels in apoptosis in these cells. To our knowledge, this is the first time it has been shown that glucocorticoid treatment reverses Trp53 activity and affects erythroid maintenance in the context of DBA. This study also demonstrates that glucocorticoid treatment of Rps19-deficient cells reverse the depletion of erythroid progenitors as well as counteracts the increase in erythroid differentiation, leading to increased expansion and CFU-E colony forming capacity (Fig 6).

Glucocorticoid treatment of DBA patients has been frequently used for decades, but comes at the cost of severe side effects. In the effort to develop more disease-specific treatments for DBA it is important to identify the specific mechanisms responsible for increased erythroid output of glucocorticoid treatment. Based on our results, it is likely that other glucocorticoid receptor ligands with dissociated functions have a more potent effect on the mechanisms relevant for DBA therapy and at the same time induce fewer side effects. This has been seen in other diseases of chronic glucocorticoid treatment, such as Duchenne muscular dystrophy (Biggar et al, 2006). The present study identifies the desired effects for a better DBA drug and is therefore a step forward toward development of more potent and safer glucocorticoid receptor agonists for DBA therapy.
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