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Survival of glucose phosphate isomerase null somatic cells and germ cells in adult mouse chimaeras

Margaret A. Keighren*, Jean H. Flockhart and John D. West

ABSTRACT

The mouse Gpi1 gene encodes the glycolytic enzyme glucose phosphate isomerase. Homozygous Gpi1−/− null mouse embryos die but a previous study showed that some homozygous Gpi1−/− null cells survived when combined with wild-type cells in fetal chimaeras. One adult female Gpi1−/−→Gpi1c/c chimaera with functional Gpi1−/− null oocytes was also identified in a preliminary study. The aims were to characterise the survival of Gpi1−/− null cells in adult Gpi1−/−→Gpi1c/c chimaeras and determine if Gpi1−/− null germ cells are functional. Analysis of adult Gpi1−/−→Gpi1c/c chimaeras with pigment and a reiterated transgenic lineage marker showed that low numbers of homozygous Gpi1−/− null cells could survive in many tissues of adult chimaeras, including oocytes. Breeding experiments confirmed that Gpi1−/− null oocytes in one female Gpi1−/−→Gpi1c/c chimaera were functional and provided preliminary evidence that one male putative Gpi1−/−→Gpi1c/c chimaera produced functional spermatozoa from homozygous Gpi1−/− null germ cells. Although the male chimaera was almost certainly Gpi1−/−→Gpi1c/c this part of the study is considered preliminary because only blood was typed for Gpi. Gpi1−/− null germ cells should survive in a chimaeric testis if they are supported by wild-type Sertoli cells. It is also feasible that spermatozoa could bypass a block at GPI, but not blocks at some later steps in glycolysis, by using fructose, rather than glucose, as the substrate for glycolysis. Although chimaera analysis proved inefficient for studying the fate of Gpi1−/− null germ cells, it successfully identified functional Gpi1−/− null oocytes and revealed that some Gpi1−/− null cells could survive in many adult tissues.

KEY WORDS: Chimaera, Chimera, Glucose phosphate isomerase, Glycolysis, Oocyte, Spermatozoa

INTRODUCTION

The dimeric glycolytic enzyme, glucose phosphate isomerase (GPI; E.C. 5.3.1.9), also known as glucose-6-phosphate isomerase, phosphoglucoisomerase or phosphohexose isomerase, catalyses the conversion of glucose-6-phosphate to fructose-6-phosphate at the second step in glycolysis and the reverse reaction during gluconeogenesis in all cell types. In mice, GPI is encoded by a single, ubiquitously expressed, autosomal gene, Gpi1, on chromosome 7.

Evidence has accumulated that several different non-enzymatic, paracrine and autocrine functions are also mediated by one or more forms of secreted, extracellular monomeric GPI, which bind to cell membrane receptors (reviewed by Henderson and Martin, 2014; Jeffery, 1999; Kim and Dang, 2005). These proteins have more restricted tissue distributions than the ubiquitous dimeric GPI enzyme and may be truncated forms of the GPI monomer with different quaternary structures (Baumann and Brand, 1988; Mizrachi, 1989; Repiso et al., 2008). The term ‘protein moonlighting’ has been coined to describe proteins, such as GPI, that can perform multiple functions (Jeffery, 1999) and databases of these proteins are now available (see Henderson and Martin, 2014).

Thus, in addition to enzymatic GPI, the Gpi1 gene encodes the neutrotrophic factor, neuroleukin, NK (Chaput et al., 1988; Faik et al., 1988; Mizrachi, 1989), the autocrine motility factor, AMF (Niinaka et al., 1998; Sun et al., 1999) and the maturation factor, MF, which is capable of mediating differentiation of leukaemia cells to monocytes (Xu et al., 1996). GPI/AMF is secreted by tumour cells, protects cells from endoplasmic reticulum stress (ER stress) and apoptosis, and promotes cell motility, epithelial to mesenchyme transition and invasion and metastasis of tumour cells (Fu et al., 2011; Funasaki et al., 2009; Kim and Dang, 2005). In addition, GPI has been identified as a specific inhibitor of myoﬁbril-bound serine proteinase in fish (Cao et al., 2000; Han et al., 2014). Finally, to confirm its remarkable ‘protein moonlighting’ multifunctional behaviour, GPI has been shown to promote embryo implantation in ferrets (Schulz and Bahr, 2003, 2004).

The mouse Gpi1α−/−β− null mutation (hereafter abbreviated to Gpi1−) is thought to alter the protein structure around the active site of the enzyme (Pearce et al., 1995). Heterozygous Gpi1−/− mice are viable and fertile but Gpi1−/− homoyzgotes fail to complete gastrulation (Kelly and West, 1996). This is likely to be solely due to the glycolytic deficiency rather than, for example, impaired epithelial to mesenchyme transition during gastrulation caused by an abnormal GPI/AMF monomer. This is because monomers have no GPI enzymatic activity and mutants that eliminate human GPI enzymatic activity do not affect the other functions of the GPI monomer (Tsutsu et al., 2003). Mouse GPI produces a testis-specific, minor isozyme (Buehr and McLaren, 1981), which appears to be a splice variant, lacking exons 5 and 6 (Vemuganti et al., 2010). However, the Gpi1− null mutation that we used produces no enzymatic activity in mouse testes (Peters and Ball, 1990). Thus, the second step of glycolysis will be blocked in male germ cells and spermatozoa as well as other cell types.

Although homozygous Gpi1−/− null mouse embryos die, the homozygous Gpi1−/− null genotype is not necessarily cell-lethal. For example, homozygous Gpi1−/−− mice were able to survive at low levels in fetal Gpi1−/−→Gpi1c/c mouse chimaeras but they contributed better to the placenta and extraembryonic endoderm...
than to fetal tissues (Kelly and West, 2002a). Similarly, tumours of GPI-deficient, Chinese hamster cells were able to grow slowly in nude mice (Pouysségur et al., 1980). Characterising to what extent cells and gametes with embryo-lethal enzyme defects, such as the homozygous Gpi1<sup>−/−</sup> genotype, can survive in mouse chimaeras may help identify how such mutant and wild-type cells interact and also help identify alternative pathways and redundancy in metabolic networks.

Although the survival of Gpi1<sup>−/−</sup> null cells has been characterised for fetal mouse chimaeras (Kelly and West, 2002a), there is only one preliminary report of an adult Gpi1<sup>−/−</sup>↔Gpi1<sup>+/+</sup> chimaera (Kelly and West, 2002b). This was a fertile female that produced oocytes, derived from Gpi1<sup>−/−</sup> null germ cells, which were capable of being fertilised and developing into fertile heterozygous Gpi1<sup>+/−</sup> offspring. However, this chimaera died so the contribution of Gpi1<sup>−/−</sup> null cells to adult tissues was not investigated in detail and no adult male Gpi1<sup>−/−</sup>↔Gpi1<sup>+/+</sup> chimaeras were produced. It remains unclear whether Gpi1<sup>+/−</sup> null cells can survive in many adult tissues and if Gpi1<sup>−/−</sup> null gametes can produce functional spermatozoa that are able to compete with wild-type spermatozoa to fertilise oocytes.

The aims of the current study were (i) to characterise the extent of survival of homozygous Gpi1<sup>−/−</sup> null cells in adult mouse chimaeras, (ii) to extend the previous preliminary study to evaluate whether female Gpi1<sup>−/−</sup>↔Gpi1<sup>+/+</sup> chimaeras can produce offspring from GPI-null oocytes and (iii) to determine whether male Gpi1<sup>−/−</sup>↔Gpi1<sup>+/+</sup> chimaeras can sire offspring from GPI-null spermatozoa derived from homozygous Gpi1<sup>−/−</sup> null germ cells.

RESULTS

Identification of adult Gpi1<sup>−/−</sup>↔Gpi1<sup>+/+</sup> chimaeras

Ninety-two adult mice were produced by aggregation of embryos, produced by the genetic crosses summarised in Fig. 1A. Chimaeras were identified initially by their variegated coat and eye pigment, and the genotype combinations were deduced from their GPI electrophoresis phenotypes. Pigment and DNA in situ hybridisation to a reiterated Tg transgenic lineage marker were used as positive markers to identify the Gpi1<sup>−/−</sup> or Gpi1<sup>+/+</sup> cells in chimaeric tissues (Keighren et al., 2015). Sixty-seven mice were overt chimaeras with variegated coat pigmentation. Fifty-seven were classified as 16 Gpi1<sup>b/b</sup>↔Gpi1<sup>a/a</sup>, 26 Gpi1<sup>a/a</sup>↔Gpi1<sup>b/b</sup> and 15 Gpi1<sup>−/−</sup>↔Gpi1<sup>−/−</sup> chimaeras by GPI electrophoresis of blood samples taken at 1, 3 and 6-7.5 months (Fig. 2A,B). The remaining ten overt coat colour chimaeras (Fig. 1C-L) produced only GPI1C (e.g. chimaeras 26 and 83 in Fig. 2A,B) and these were provisionally classified as Gpi1<sup>−/−</sup>↔Gpi1<sup>−/−</sup> chimaeras. At this stage it remained possible that some were other genotype combinations with more than 97% GPI1C in the blood sample as less than 3% of one GPI1 allozyme may not always be detected (Kelly and West, 2002a). This was only likely for chimaeras 83 and 89 which both had only approximately 5% coat pigmentation and 3% Tg-positive nuclei in blood smears. However, all ten chimaeras were confirmed as being Gpi1<sup>−/−</sup>↔Gpi1<sup>−/−</sup> chimaeras by post-mortem analysis of other tissues (described below), breeding studies (chimera 83) or both (chimera 22). Although the frequency of overt chimaeras that were identified as Gpi1<sup>−/−</sup>↔Gpi1<sup>−/−</sup> chimaeras (10/67=14.9%) was lower than the expected frequency of 25%, this was not significant by a chi square goodness-of-fit test (P=0.078).

Of the remaining 25 mice that were not coat colour chimaeras, four were uniformly pigmented (1 Gpi1<sup>b/b</sup>, 1 Gpi1<sup>a/a</sup> and 2 Gpi1<sup>−/−</sup>) and 21 were entirely albino with only GPI1C detected in the blood. Some of these albino, GPI1C mice may have been cryptic chimaeras (including Gpi1<sup>−/−</sup>↔Gpi1<sup>−/−</sup> chimaeras) with a minor cell population that was excluded from coat and eye pigment and below detectable limits in the blood but this was not investigated further. Also, some may have been Gpi1<sup>−/−</sup>↔Gpi1<sup>−/−</sup> chimaeras, from which the Gpi1<sup>−/−</sup> cell population was eliminated by 1 month after birth.

Test breeding to identify chimaeras with functional Gpi1<sup>−/−</sup> null germ cells

To investigate whether any of the ten Gpi1<sup>−/−</sup>↔Gpi1<sup>−/−</sup> chimaeras could produce functional gametes from homozygous Gpi1<sup>−/−</sup> null germ cells, they were crossed to albino Gpi1<sup>+/−</sup> mice, as shown in Fig. 1B. Chimaera 53 developed a tumour and was culled when it was pregnant with its first litter; all eight fetuses had unpigmented eyes and were GPI1C (expected genotype, Gpi1<sup>−/−</sup>). Seven other chimaeras each produced at least 45 first generation (G1) offspring, none of which was pigmented. At least three albino G1 offspring of each of these 7 chimaeras were typed for GPI1 and all were GPI1C (expected genotype, Gpi1<sup>−/−</sup>). Female chimaera 22 and male chimaera 83 produced both albino and pigmented offspring.

Female chimaera 22 produced four litters with 15/46 (32.6%) pigmented offspring overall, as shown in Fig. 3A. All 15 pigmented G1 offspring and the 9 albino, G1 offspring that were tested (from each of three litters) were GPI1C. Albino GPI1C, G1 offspring were expected to be Gpi1<sup>b/b</sup> homozygotes but pigmented GPI1C offspring were expected to be Gpi1<sup>+/−</sup> heterozygotes (Fig. 1B). The probability of all 15 pigmented G1 offspring being GPI1C if the chimaera was either Gpi1<sup>b/b</sup>↔Gpi1<sup>−/−</sup> or Gpi1<sup>+/−</sup>↔Gpi1<sup>−/−</sup> is only (1/2)<sup>5</sup> (i.e. P=0.00003), which is very strong evidence that chimaera 22 was a Gpi1<sup>−/−</sup>↔Gpi1<sup>−/−</sup> chimaera. To check that all 15 pigmented G1 offspring were actually Gpi1<sup>−/−</sup> heterozygotes, they were crossed to pigmented, Gpi1<sup>b/b</sup> mice, as explained in Fig. 1B, and each produced at least two litters. The second generation (G2) offspring were all pigmented and were typed for GPI to check that all G1 offspring produced both GPI1B (Gpi1<sup>b/b</sup>) and GPI1BC (Gpi1<sup>b/c</sup>) G2 offspring and that approximately equal numbers were produced overall. In total, 122 G2 offspring were GPI1B and 120 were GPI1BC and Fig. 3B shows that all 15 G1 mice produced both GPI1B and GPI1BC G2 offspring. This implied that all the pigmented GPI1C G1 mice were Gpi1<sup>b/c</sup> rather than Gpi1<sup>−/−</sup>. The genetic crosses showed that chimaera 22 was a genuine Gpi1<sup>−/−</sup>↔Gpi1<sup>−/−</sup> chimaera with homozygous Gpi1<sup>−/−</sup> null germ cells that produced functional oocytes, which were capable of producing viable offspring when fertilised by wild-type spermatozoa. The composition of various body tissues in this chimaera is discussed below.

Male chimaera 83 produced 12 litters with 6/93 (6.5%) pigmented offspring overall (Fig. 3C). All six pigmented G1 offspring and the 18 albino G1 offspring tested (from six litters) were GPI1C. The probability of all 6 pigmented G1 offspring being GPI1C if the chimaera was either Gpi1<sup>b/b</sup>↔Gpi1<sup>−/−</sup> or Gpi1<sup>+/−</sup>↔Gpi1<sup>−/−</sup> is only (1/2)<sup>6</sup> (i.e. P=0.00156), which is good evidence that chimaera 83 was a Gpi1<sup>−/−</sup>↔Gpi1<sup>−/−</sup> chimaera. The 6 pigmented G1 offspring were crossed to pigmented, Gpi1<sup>b/b</sup> mice and each produced at least two litters. In total, there were 40 GPI1B and 40 GPI1BC G2 offspring and all six G1 mice produced both GPI1B and GPI1BC G2 offspring (Fig. 3D). This confirms that the pigmented GPI1C G1 mice were all Gpi1<sup>b/c</sup> rather than Gpi1<sup>−/−</sup>. The genetic crosses imply that chimaera 83 was a genuine Gpi1<sup>b/c</sup>↔Gpi1<sup>b/c</sup> chimaera with homozygous Gpi1<sup>−/−</sup> null germ cells that produced functional spermatozoa, which were capable of fertilising wild-type oocytes and producing viable offspring. Unfortunately, this chimaera died at 6 months and tissues were not available for further analysis, so the genotype classification is
based on the presence of a pigmented cell population, GPI electrophoresis of the blood and the absence of Gpi1<sup>−/−</sup> or Gpi1<sup>c/c</sup> mice among the six pigmented, G1 offspring. On this basis, male chimaera 83 was almost certainly a Gpi1<sup>−/−</sup>↔Gpi1<sup>c/c</sup> chimaera. However, as only blood was genotyped for GPI, this part of the study should be considered preliminary.
Contribution of homozygous Gpi1<sup>−−</sup> null cells to chimaeras

Chimaeras were killed at 6-7.5 months of age, after the test breeding was completed. Eyes were checked for pigment and a subjective estimate of the percentage of eye pigmentation was made for each eye for all the chimaeras. The initial genotype assignments, based on GPI electrophoresis of blood, were checked by GPI electrophoresis of the different body tissues and organs listed in the Materials and methods (Fig. 2C-J). Samples were analysed from nine Gpi1<sup>−−</sup>↔Gpi1<sup>c/c</sup> chimaeras (including chimaera 53, which was culled before 6 months, but not chimaera 83, which died). All samples from all nine chimaeras only produced GPI1C bands, confirming that they were all Gpi1<sup>−−</sup>↔Gpi1<sup>c/c</sup> chimaeras.

There was a trend for body mass of male Gpi1<sup>−−</sup>↔Gpi1<sup>c/c</sup> chimaeras to be lighter than those in the other groups and for some comparisons this was significant at 3 and 6-7.5 months (Fig. 3E-G), suggesting that growth was affected. Female chimaeras were excluded from these comparisons as some were pregnant.

Comparisons of subjective estimates of the percentage coat and eye pigmentation and the percentage of Tg-positive nucleated blood cells at 3 months all showed that Gpi1<sup>−−</sup> null cells contributed much less than Gpi1<sup>a/b</sup>, Gpi1<sup>a−</sup> or Gpi1<sup>b−</sup> cells to chimaeras (Fig. 3H-J). This strongly suggests that, for these tissues at least, Gpi1<sup>−−</sup> null cells were at a selective disadvantage, as previously reported for Gpi1<sup>−−</sup>↔Gpi1<sup>c/c</sup> fetal chimaeras (Kelly and West, 2002a).

Pigmented Gpi1<sup>−−</sup> null cells tended to form radial stripes in the iris (Fig. 4H) and large patches in the choroid of Gpi1<sup>−−</sup>↔Gpi1<sup>c/c</sup> chimaeric eyes (Fig. 4GJeU), as reported for other chimaeras and mosaics (Gordon, 1977; West, 1976). Spatial distributions of pigmented Gpi1<sup>−−</sup> null cells could also be seen in the retinal pigment epithelium (RPE) of intact eyes of Gpi1<sup>−−</sup>↔Gpi1<sup>c/c</sup> chimaeras if the overlying choroid was largely unpigmented (Fig. 4U-Y). Near the RPE periphery, some patches of pigmented RPE cells formed radial stripes in chimaera 22 (Fig. 4U), similar to those reported for other pigmented ↔albino chimaeras and mosaics (Bodenstein and Sidman, 1987; Collinson et al., 2004; Hodson et al., 2011). In eyes of Gpi1<sup>−−</sup>↔Gpi1<sup>c/c</sup> chimaeras with lower proportions of pigmented Gpi1<sup>−−</sup> null cells, pigmented cells formed small clusters or discontinuous stripes in the RPE (Fig. 4V-Y).

DNA in-situ hybridisation (ISH) was used to detect the Tg lineage marker in several tissues of Gpi1<sup>−−</sup>↔Gpi1<sup>c/c</sup> chimaeras and examples of tissues with Tg-positive cells are shown in Fig. 5 for...
Fig. 3. Chimaera breeding experiments, body mass and composition. (A-D) Results of breeding experiments with two putative Gpi1\(^{-/-}\) \(\leftrightarrow\) Gpi1\(^{c/c}\) chimaeras, showing production of some pigmented (putative Gpi1\(^{c/-}\)), G1 generation offspring when crossed to albino, Gpi1\(^{c/c}\) mice (A,C) and confirmation that all pigmented, G1 offspring produced both GPI1B (Gpi1\(^{b/-}\)) and GPI1BC (Gpi1\(^{b/c}\)) offspring in generation G2, when crossed to Gpi1\(^{b/b}\) mice (B,D), as outlined in Fig. 1B. (E-G) Comparisons of body mass of male chimaera of four genotypes at 1 month (E), 3 months (F) and 6-7.5 months (G). Genotypes were compared by one-way ANOVA, (\(P\)-values are shown on the graphs) and Tukey’s multiple comparison test (asterisks). Females were not included as some were pregnant at 3 and 6-7.5 months. (H-J) Comparisons of composition of chimaera of four genotypes from subjectively estimated percentage coat pigmentation (mean of estimates at 1, 3 and 6-7.5 months) (H), subjectively estimated percentage eye pigmentation (mean of left and right eyes) (I) and percentage of Tg-positive nuclei in blood smear at 3 months (J). Genotypes were compared by Kruskal-Wallis test (\(P\)-values are shown) and Dunn’s multiple comparison test (asterisks). Means are shown by horizontal bars. *\(P<0.05\); **\(P<0.01\); ***\(P<0.001\).
female chimaera 22, which produced pigmented offspring in the genetic crosses. This includes a Tg-positive oocyte in a pre-antral ovarian follicle (Fig. 5A), which is consistent with the genetic evidence that chimaera 22 produced $Gpi1^{−/−}$ null oocytes. There were many Tg-positive mural granulosa cells in the part of the antral ovarian follicle shown in Fig. 5B but there were few Tg-positive cells elsewhere in the section. Tg-positive cells were abundant in some regions of sections of thymus (Fig. 5C) and spleen (not shown) from this particular chimaera but this was not studied quantitatively. In other tissues, Tg-positive cells tended to occur in isolation or in small clusters (Fig. 5D,E). The small group of Tg-positive cells in the adrenal cortex, shown in Fig. 5D, appeared to be radially aligned across the cortex as reported for other chimaeras and mosaics (Morley et al., 2004, 1996; Weinberg et al., 1985; West, 2001).
group of Tg-positive cells may have been produced by a Tg-positive, Gpi1−/− null stem cell as there is evidence that stem cells are located in the outer adrenal cortex and produce daughter cells that move inwards towards the medulla (Chang et al., 2013; King et al., 2009; Wood et al., 2013). In adult mice, the epithelium of intestinal villi is maintained by stem cells in the crypts and eventually each crypt harbours a single clone of stem cells (Ponder et al., 1985; Snippert et al., 2010). The section across several intestinal villi, in Fig. 5F, shows that epithelia of several villi are largely Tg-positive whereas others are Tg-negative, suggesting that Tg-positive, Gpi1−/− null stem cells are capable of maintaining intestinal villi.

Gpi1−/− null cells, identified either as pigmented or Tg-positive cells, were present in all the tissues and organ samples investigated in two females among the five female and four remaining male Gpi1−/−↔Gpi1c/c chimaeras analysed (Fig. 6A). However, we did not identify any Tg-positive cells in sections of testis that were examined from the four surviving male chimaeras. The other putative male Gpi1−/−↔Gpi1c/c chimaera was chimaera 83, which died before GPI genotyping of blood was confirmed with other tissues. If the preliminary genotype assignment for chimaera 83 is correct, the breeding results would imply that Gpi1−/− male germ cells could survive in the testis. Although each of the nine Gpi1−/−↔Gpi1c/c chimaeras that were analysed had Gpi1−/− null cells in multiple tissues or organs, the frequency of samples with detectable Gpi1−/− null cells varied among chimaeras. The four Gpi1−/−↔Gpi1c/c chimaeras that were estimated to have at least 20% pigment in the coat had a significantly higher frequency of eye samples (RPE, choroid and iris in sections of left and right eyes) with pigment (20/24; 83%) than in the five chimaeras that were estimated to have less than 20% coat pigment (9/30; 30%; Fisher’s exact test P=0.00011). Similarly, for the samples analysed by in situ hybridisation, there were significantly more samples with the Tg-marker in the chimaeras with at least 20% coat pigment (54/61; 89%) than in the chimaeras with less than 20% coat pigment (48/79; 61%; Fisher’s exact test P=0.00024).

The percentages of Tg-positive nuclei were estimated from counts in blood smears and sections of kidneys, adrenal glands and ovaries for the nine surviving Gpi1−/−↔Gpi1c/c chimaeras and five control chimaeras (Gpi1d/b↔Gpi1c/c, Gpi1d−/−↔Gpi1c/c or Gpi1d+/−↔Gpi1c/c) that were judged to have no more than 30% coat pigment. Although the five control chimaeras were those with the lowest coat pigment they still had more coat pigment (mean=22.0%; range=10-30%) than those of the Gpi1−/−↔Gpi1c/c chimaeras (mean=13.3%; range=2-23%). The quantitative comparisons (Fig. 6B) showed that Gpi1−/− null cells made a

Fig. 5. Tg-positive Gpi1−/− null cells in different tissues of Gpi1−/−↔Gpi1c/c chimaera 22. The Tg marker is identified as a small brown in situ signal (arrows) within some nuclei and the tissue sections are weakly stained with haematoxylin and eosin. The in situ signal is often not in the same plane of focus as the tissue section, so the cells sometimes appear out of focus. (A) Tg-positive oocyte in an ovarian pre-antral follicle. (B) Part of an ovarian antral follicle with abundant Tg-positive cells in the mural granulosa cell layer. (C) Region of thymus with abundant Tg-positive cells. (D) Adrenal cortex showing a single Tg-positive cell within the outer zona granulosa layer (top arrow) and a line of several Tg-positive cells in the zona fasciculata layer of the cortex (between other two arrows). (E) Adrenal medulla showing five Tg-positive cells (arrowed). (F) Cross section of small intestinal villi showing several villi with abundant Tg-positive cells on the left and other villi with no Tg-positive cells. Scale bar=20 µm.
very low contribution to these tissues in all the $Gpi1^{-/-}$ null chimaeras and this was probably comparable to the lowest contributions of wild-type cells in control chimaeras. This provides further evidence for selection against homozygous $Gpi1^{-/-}$ null cells in $Gpi1^{-/-}$ null chimaeras.

To test whether selection against $Gpi1^{-/-}$ null nucleated blood cells continued in adults, the percentages of Tg-positive $Gpi1^{-/-}$ null cells in blood smears taken at 1, 3 and 6-7.5 months were compared for the seven $Gpi1^{-/-}$ null chimaeras that were analysed at each of these three time points (Fig. 6C). A Friedman test for repeated measures showed no overall differences among ages but Dunns multiple comparison tests indicated there was a reduction in the contribution of Tg-positive cells between 3 and 6-7.5 months. In principle, this could be attributable to selection against the $Gpi1^{-/-}$ null genotype, differences between the genetic backgrounds of the $Gpi1^{-/-}$ null cells and $Gpi1^{-/-}$ null cells in the chimaera or stochastic variation. However, no such change occurred between 1 and 3 months so there is no convincing evidence for ongoing selection against homozygous $Gpi1^{-/-}$ null nucleated blood cells between 1 and 6-7.5 months.

Overall, it is clear that homozygous $Gpi1^{-/-}$ null cells make a very low contribution to all the tissues and organs of adult chimaeras that were tested and, in some individual samples, no $Gpi1^{-/-}$ null cells were identified. Although this suggests that $Gpi1^{-/-}$ null cells are at a general selective disadvantage there was no evidence that they were consistently excluded from specific tissues.

**Contribution of heterozygous $Gpi1^{+/a}$ and $Gpi1^{+/b}$ cells to chimaeras**

Although it was not possible to use quantitative GPI electrophoresis to investigate the contribution of $Gpi1^{-/-}$ null cells to...
shown) and Dunn percentage GPI1C was corrected for reduced GPI1 production from chimaeras from (A) mean composition of eight samples (brain, right kidney, medial liver lobe, heart, spleen, small intestine, pancreas and lung), (B) mean composition of three samples (brain, kidney and liver), that were selected to determine derivatives of the ectoderm mesoderm and endoderm respectively (Fig. 7A,B). As the percentage of GPI1C was not representative of GPI1CC instability (2× corrected). In C, the observed percentage GPI1C was corrected, both for the reduced composition of final blood sample (6-7.5 months). In A and B, the observed percentage GPI1C produced by Gpi1a/b−/− cells, as in principle, this pathway could by-pass the block in glycolysis at GPI (Fig. 8A,B). The pentose phosphate pathway begins with glucose-6-phosphate and, if it generates surplus ribose-5-phosphate, some is converted to fructose-6-phosphate and glyceraldehyde 3-phosphate. However, presumably neither the TCA cycle nor the pentose phosphate pathway can fully compensate for the GPI deficiency, otherwise Gpi1−/− null embryos would survive and Gpi1−/− null cells would not be severely depleted in chimaeras.

Some Gpi1−/− null cells in chimaeric tissues are probably rescued by neighbouring wild-type cells or blood, which could provide ATP or substrates for glycolysis downstream of GPI. The wild-type cells may not always need to be in the same tissue to support Gpi1−/− null...
cells. For example, the retinal pigment epithelium is a monolayer so \textit{Gpi1}\textsuperscript{−/−} null RPE cells could be supported by wild-type cells in the adjacent neural retina or choroid as well as the RPE itself. The survival of \textit{Gpi1}\textsuperscript{−/−} null nucleated blood cells (Tg-positive cells in blood smears) indicates that continuous, direct contact with neighbouring wild-type cells is not essential for survival of all \textit{Gpi1}\textsuperscript{−/−} null cells.

Previous evidence from fetal chimaeras showed that selection against \textit{Gpi1}\textsuperscript{−/−} null cells begins before E12.5 (Kelly and West, 2002a) but it is not known whether selection pressure decreases once
there are only a small number of Gpi1<sup>−/−</sup> null cells that are scattered individually or in small groups among many more wild-type cells. Although no systematic spatial analysis was carried out, the distribution of Gpi1<sup>−/−</sup> null cells appeared to follow this pattern in some tissues. The depletion of Gpi1<sup>−/−</sup> null cells in chimaeras implies that they are at a selective disadvantage, at least during development, and that this glycolytic deficiency acts as a cell-autonomous defect. In both the present study and the earlier pilot study (Kelly and West, 2002b), a Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaera died of unknown causes before 6 months but it is not known whether this is just chance or if the presence of Gpi1<sup>−/−</sup> null cells affects the fitness of chimaeras.

**Production of functional oocytes from Gpi1<sup>−/−</sup> null germ cells in mouse chimaeras**

In the breeding experiments, only 2/10 putative Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaeras (one female and one male) produced offspring from the pigmented, Gpi1<sup>+/−</sup> germ cell population. This is not surprising, however, because the albino, Gpi1<sup>+/−</sup> cell population predominated in all the Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaeras. Fifty percent of Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaeras are expected to be XX↔XY chimaeras, which are predicted to produce only albino offspring. This is because male XX↔XY chimaeras will only produce functional XY germ cells and female XX↔XY chimaeras will only produce functional XX germ cells (McLaren, 1975; Mullen and Whitten, 1971). In each case, these are expected to be genetically albino because the predominant somatic cell population of the developing gonad determines the sex of XX↔XY chimaeras. In these chimaeras the predominant cell population will be genetically albino and Gpi1<sup>+/−</sup>. The remaining 50% of Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaeras will be XX↔XX and XY↔XY chimaeras and could produce both pigmented and albino offspring. However, genetically albino, Gpi1<sup>+/−</sup> germ cells are likely to predominate so most offspring are expected to be albino.

Analysis with the Tg lineage marker showed that some Gpi1<sup>−/−</sup> null oocytes and ovarian follicle cells survived in several female Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaeras. Breeding experiments also showed that one of these adult female Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaeras produced offspring, which must have been generated by the fertilisation of Gpi1<sup>−/−</sup> null oocytes. Together with the previous preliminary report (Kelly and West, 2002b) this means that two adult female Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaeras have now been identified that produced functional Gpi1<sup>−/−</sup> null oocytes, which were fertilised and developed into fertile heterozygous Gpi1<sup>+/−</sup> offspring. Mouse oocytes produce energy by metabolising pyruvate via the TCA cycle whereas ovarian follicle cells mainly rely on glycolysis and can secrete pyruvate (Biggers et al., 1967; Boland et al., 1994; Donahue and Stern, 1968; Downs et al., 2002; Downs and Mastropolo, 1994; Downs and Utecht, 1999; Leese and Barton, 1985). Thus, wild-type follicle cells in chimaeric oocytes could rescue Gpi1<sup>−/−</sup> null oocytes by providing them with pyruvate or ATP and may also rescue neighbouring Gpi1<sup>−/−</sup> null follicle cells by providing them with ATP or intermediate glycolytic metabolites downstream of the GPI block at step 2 of glycolysis as discussed previously (Kelly and West, 2002b).

**Production of functional spermatozoa from Gpi1<sup>−/−</sup> null germ cells in mouse chimaeras**

No adult male Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaeras had been identified previously, so one aim of the present study was to determine if male Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaeras had Gpi1<sup>−/−</sup> null germ cells that produced functional spermatozoa. The GPI composition of the blood of one male chimaera and the genetic breeding results implied that this was almost certainly a Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaera. However, this mouse died and other tissues were not available to confirm the GPI genotype assignment, so this provides only preliminary evidence that homozygous Gpi1<sup>−/−</sup> null germ cells can produce functional spermatozoa.

As we identified one male that was almost certainly a Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaera, we need to consider how homozygous Gpi1<sup>−/−</sup> null spermatozoa could survive in the chimaeric testis and generate functional, GPI-null spermatozoa that compete successfully, with wild-type spermatozoa in the female reproductive tract, to fertilise oocytes. Functional, haploid Gpi1<sup>−/−</sup> null spermatozoa are produced routinely by germ cells in heterozygous Gpi1<sup>+/−</sup> males. However, this is readily explained because the progeny of each A-type paired spermatagonia form a large syncytium of developing germ cells that are connected by cytoplasmic bridges (Greenbaum et al., 2011). The cytoplasmic bridges are large enough to allow exchange of cytoplasm, including mRNA, protein and even organelles (Ventela et al., 2003), so that genetically haploid sperm are considered to be phenotypically diploid (Braun et al., 1989). Survival of genetically haploid Gpi1<sup>−/−</sup> null spermatozoa produced by heterozygous Gpi1<sup>+/−</sup> males can therefore, be explained because the spermatozoa will be phenotypically equivalent to diploid Gpi1<sup>−/−</sup> cells. In contrast, haploid Gpi1<sup>−/−</sup> null spermatozoa produced by homozygous Gpi1<sup>−/−</sup> null germ cells in a chimaera will be phenotypically equivalent to homozygous, diploid Gpi1<sup>−/−</sup> null cells because all the interconnected germ cells will be derived from the same homozygous Gpi1<sup>−/−</sup> null spermatogonium. Thus, we need to consider how the GPI block to glycolysis could be overcome by Gpi1<sup>−/−</sup> null germ cells in the chimaeric testis and by phenotypically GPI-null, haploid Gpi1<sup>−</sup> spermatozoa in the female reproductive tract.

Sertoli cells support spermatogonia, spermatocytes and spermatids in the testis and generate lactate from glucose via glycolysis and lactate dehydrogenase (Jutte et al., 1983; Mita and Hall, 1982; Robinson and Fritz, 1981). Spermatocytes and round spermatids do not utilise glucose but convert lactate, secreted by the Sertoli cells, to pyruvate which produces energy via the TCA cycle and oxidative phosphorylation (Jutte et al., 1982, 1983; Mita and Hall, 1982; Nakamura et al., 1984, 1986). Thus, GPI-null spermatogonia, spermatocytes and spermatids should survive if they are supported by wild-type Sertoli cells, which will predominate in Gpi1<sup>−/−</sup>↔Gpi1<sup>+/−</sup> chimaeras. The survival of Gpi1<sup>−/−</sup> null somatic cells and germ cells in chimaeras may be wholly dependent on the presence of neighbouring wild-type cells. However, ejaculated spermatozoa, produced by male Gpi1<sup>−/−</sup> null germ cells, are not constantly in contact with wild-type cells, so their survival will depend on whether nutrients and alternative metabolic pathways are available to by-pass the block to glycolysis at GPI.

Glycolysis becomes important when spermatozoa reach the cauda epididymis, where they become motile, and glycolysis continues to be important after ejaculation (Miki, 2007). Both glycolysis and the TCA cycle are active in ejaculated spermatozoa but ATP production is compartmentalised. Enzymes that produce ATP by the TCA cycle are in the mitochondria of the mid-piece but enzymes that produce ATP by glycolysis are localised to the head and principal piece (du Plessis et al., 2015; Krisfalusi et al., 2006; Mukai and Travis, 2012; Westhoff and Kamp, 1997). Glucose is supplied to spermatozoa by the fluid of the female reproductive tract.
and from the spermatozoa’s own glycogen stores (Ballester et al., 2000) and it is the substrate for both glycolysis and the pentose phosphate pathway, which generates NADPH. Seminal fluid is rich in fructose, which can replace glucose as the substrate for glycolysis (Fig. 8A,C) but cannot replace glucose in the pentose phosphate pathway (Fraser and Quinn, 1981; Goodson et al., 2012).

Different steps in fertilisation require ATP, generated by glycolysis or the TCA cycle, and NADPH, generated via the pentose phosphate pathway (Miki, 2007; Urner and Sakkas, 2003, 2005). Glycolysis is required for progressive motility of spermatozoa and, in vitro experiments show that motility can be supported by glycolysis, fuelled by glucose, fructose, mannose or sorbitol (Goodson et al., 2012). Capacitation of spermatozoa involves an increase in membrane fluidity, induction of hyperactivation and tyrosine phosphorylation of proteins (Naz and Rajesh, 2004; Visconti et al., 1995). This requires glycolysis and the pentose phosphate cycle (Aquila et al., 2010; Goodson et al., 2012; Miraglia et al., 2010) so it normally depends on glucose but tyrosine phosphorylation can occur in vitro if glucose is replaced by fructose (Goodson et al., 2012). The acrosome reaction requires lactate or pyruvate to drive the TCA cycle but does not require glucose (Miki, 2007; Urner and Sakkas, 1996) and sperm-oocyte fusion requires the pentose phosphate pathway (Urner and Sakkas, 2005).

GPI-null spermatozoa would be able to generate ATP by the TCA cycle but sperm motility may depend on local production of ATP by glycolysis in the principal piece (du Plessis et al., 2015; Westhoff and Kamp, 1997). GPI-null spermatozoa would also have an intact pentose phosphate pathway, so NADPH production should be unaffected (Fig. 8A,B). This pathway might also help by-pass the GPI block to glucose-fuelled glycolysis but fructose-fuelled glycolysis would probably be more effective. In principle, fructose might generate energy via glycolysis, after by-passing GPI, by one of two pathways (Fig. 8A,C). Although hexokinase has a lower affinity for fructose than for glucose, it can convert fructose to fructose-6-phosphate, which enters glycolysis, at step 3, having by-passed GPI. Alternatively, fructokinase can convert fructose to fructose-1-phosphate, which enters glycolysis at step 6 via the Hersh pathway (Fraser and Quinn, 1981; Goodson et al., 2012; Hers, 1955).

Neither fructose-fuelled glycolysis nor the pentose phosphate pathway would by-pass blocks further down the glycolytic pathway. This is consistent with the infertility or sub-fertility of Gapdh−/−, Pgi2−/− and Enol−/− knockout male mice, which lack testis-specific forms of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and enolase, respectively (Danishina et al., 2010; Miki et al., 2004; Nakamura et al., 2013). In sperm, these enzymes are required for glycolysis steps 6, 7 and 9 (Fig. 8A). Male infertility or sub-fertility also occurs in Ldhc−/− knockout mice that lack testis-specific lactate dehydrogenase enzyme, which interconverts pyruvate (the final product of glycolysis) and lactate (Odet et al., 2008, 2011). Thus, if fructose acted as a substrate for glycolysis, this should bypass the GPI block at step 2 of glycolysis in phenotypically GPI-null, haploid Gpi1−/− null spermatozoa that are produced by homozygous Gpi1−/− null germ cells.

Conclusions

Chimaera analysis proved to be an inefficient approach to study the fate of Gpi1−/− null germ cells as only two of ten Gpi1−/−→Gpi1+/c chimaeras produced offspring from the genetically pigmented, Gpi1−/− cell population. A more detailed investigation of the rescue of Gpi1−/− null, male germ cells and the phenotypically GPI1 null spermatozoa that they produce requires other approaches. For example, Gpi1 could be conditionally knocked out, specifically in male germ cells using Cre-loxP transgenic mice with a spermatocyte-specific or spermatid-specific Cre-driver (Smith, 2011). Nevertheless, our experiments with adult Gpi1−/−→Gpi1+/c chimaeras showed that, although Gpi1−/− null cells are at a selective disadvantage, some could survive in adult somatic tissues. These are likely to be rescued by neighbouring wild-type cells. Genetic breeding experiments supported the previous, preliminary report that Gpi1−/− null oocytes can survive and be fertilised and they also provided preliminary evidence that homozygous Gpi1−/− null germ cells can produce functional spermatozoa. Wild-type follicles are thought to support Gpi1−/− oocytes and wild-type Sertoli cells are likely to support Gpi1−/− spermatozoa, spermocytes and spermatids. We suggest that, for phenotypically GPI-null spermatozoa, the deficiency in glucose-fuelled glycolysis may be by-passed by fructose-fuelled glycolysis. This is only feasible because GPI is an early step in glycolysis and defects in enzymes required for later steps of glycolysis would not be rescued in this way.

MATERIALS AND METHODS

Mice

All work with mice (Mus musculus Linnaeus) was performed in accordance with institutional guidelines and UK Home Office regulations (licences PPL 60/1150 and PPL 60/1989). Mice were housed under conventional conditions in the University of Edinburgh, Medical School. Two pigmented (Tyr+/c) stocks (designated ‘GN’ and ‘NUL’), carrying the Gpi1a−/− and Gpi1b−/− null allele (Pearce et al., 1995; Peters and Ball, 1990), were maintained as Gpi1a¹⁺/¹⁺−/− and Gpi1b¹⁺/¹⁺−/−, respectively. Gpi1b−/− males were used as GDH−/− (Abbott et al., 1988; Lo, 1986; Lo et al., 1987), which we used as a target for DNA hybridisation (Keighren and West, 1993). The abbreviation Tg/− denotes mice without the reiterated transgene, Tg+/− denotes hemizygotes and Tg+c/c denotes homozygotes.

Chimaera production

Adult mouse chimaeras (series AdCK) were produced by aggregating pairs of preimplantation embryos (Tarkowski, 1961), as described elsewhere (Kelly and West, 2002a,b). Stock Gpi1a/b−/− null oocytes can survive and be fertilised and they also develop to Gpi1a/b−/−→Gpi1a+b−/− mule mice in alternate generations as previously described (Kelly and West, 2002a,b). Stock ‘NUL’ was homozygous (Tg/−) for the reiterated β-globin transgene lineage marker Tg(NHh-b)83Clo (Katsuura and Lo, 1988; Lo, 1986; Lo et al., 1987), which we used as a target for DNA in situ hybridisation (Keighren and West, 1993). The abbreviation Tg/− denotes mice without the reiterated transgene, Tg+/− denotes hemizygotes and Tg+c/c denotes homozygotes.

Analysis of chimaeras

Putative chimaeras were weighed and the percentage coat pigmentation was estimated subjectively. Blood smears and samples of some solid tissues and organs were prepared for histology and DNA in situ hybridisation as described below. Other samples were homogenised in distilled water with a Polytron homogeniser and stored at −20°C in 1.5 ml.
microtubes for GPI electrophoresis. Samples analysed by GPI electrophoresis included blood, brain, heart, spleen, left and right kidneys, four liver lobes (medial, left lateral, right lateral and caudal), pancreas, small intestine (duodenum), lung, muscles from all four limbs, tongue and either seminal vesicle or the right uterine horn.

**GPI electrophoresis**

Cellulose acetate electrophoresis and staining for GPI activity was carried out as described previously (West and Flockhart, 1994) to separate the GPI1C allozyme band (GPI1CC homodimer), encoded by the Gpi1c allele, from GPI1A, GPI1AB and GPI1B allozyme bands, encoded by the Gpi1a and Gpi1b alleles. Blood samples were used for initial genotype assignments and this was checked by electrophoresis of a range of tissue samples collected post-mortem. Images of the stained electrophoresis plates were obtained using a flatbed scanner (Epson V330 photo), cropped using Adobe Photoshop CS6 software (Adobe Systems Inc. San Jose, CA) and converted to high-contrast, greyscale images using the auto contrast function.

To estimate the percentage contribution of Gpi1c in Gpi1b+/Gpi1c–, Gpi1c–/Gpi1c– and Gpi1b+/Gpi1c– chimaeras, the percentage GPI1C band was estimated in a selected group of tissues and organs by scanning densitometry (West and Flockhart, 1994). However, the percentage GPI1C band was estimated in a selected group of tissues and sample mixtures was 34.62±3.04% for mixtures of Gpi1b+/Gpi1c–, Gpi1c–/Gpi1c– and Gpi1b+/Gpi1c– mice. Each of three mixtures (Gpi1b+/Gpi1c+, Gpi1c+/Gpi1c+ and Gpi1b+/Gpi1c–) was prepared three times (using different mice each time) for eight samples (brain, right kidney, medial liver lobe, heart, spleen, small intestine, pancreas and lung).

Although most tissues of chimaeras show an additive banding pattern of the two constituent genotypes, skeletal muscle samples (including tongue) produce additional bands. This is because muscle development involves fusion of myoblasts (Mintz and Baker, 1967) and GPI is a dimer. If myoblasts of two constituent genotypes, skeletal muscle samples (including tongue) but skeletal muscle also produced AC combinations can occur. For example, most tissues of chimaeras were fixated in acetic alcohol, after fixation, lenses were also included as quality controls in each in situ hybridisation run.

**Histology and DNA in situ hybridisation**

Blood samples were air-dried on clean microscope slides, fixed in acetic alcohol (3 ethanol:1 acetic acid, v/v) for 60 min, air dried, immersed in acetone for 10 min and dehydrated through graded alcohols before in situ hybridisation (ISH). Tissue samples for DNA ISH or eye histology for pigment analysis were fixed in acetic alcohol. After fixation, lenses were removed from the eyes, through a cut made in the cornea, to facilitate sectioning. Samples of solid tissues were processed to paraffin wax for histology. Sections were cut at 7 µm thickness and mounted on glass microscope slides coated with 3-aminopropyltriethoxysilane (TESPA; Sigma-Aldrich, Poole, UK). Tissue sections and blood smears were analysed by DNA ISH to the transgene and hybridised digoxygenin-labelled DNA probe was detected by diaminobenzidine (DAB) staining for peroxidase-labelled antibody as described previously (Keighren and West, 1993). Slides were counterstained with haematoxylin and eosin and examined by bright-field microscopy to identify Tg<sup>+</sup> cells derived from the Gpi1<sup>b</sup>–/Gpi1<sup>c</sup>–, Gpi1<sup>b</sup>–/Gpi1<sup>c</sup>– or Gpi1<sup>c</sup>– (GN×NUL) component in the chimaera by the presence of a brown hybridisation signal in the nucleus.

For most tissues in the chimaeras, the contribution of Tg-positive cells was scored qualitatively as positive or negative for the presence of nuclei containing the hybridisation signal after ISH. Tissue sections with Tg-positive nuclei were photographed using a calibrated Zeiss Axiosvision 4.8 digital camera system on a Zeiss Axiosplan 2 compound microscope. Quantitative counts of Tg-positive nuclei were made for blood smears and sections of kidneys, adrenals and ovaries using a Leica Dianplan compound microscope with a 10×10 eyepiece grid. Crude counts of Tg-positive nuclei were corrected using the percentage of Tg-positive nuclei seen in the equivalent tissues from hemizygous Tg<sup>+</sup> positive control mice. For each tissue section or blood smear, approximately 300 nuclei were scored for the presence of the hybridisation signal. Tg<sup>+</sup> negative control sections were also included as quality controls in each in situ hybridisation run.

**Test breeding to evaluate gamete function**

To test whether putative Gpi1<sup>c</sup>–/Gpi1<sup>c</sup>– chimaeras contained a Gpi1<sup>c</sup>–/Gpi1<sup>c</sup>– null germ cell population that could produce functional gametes, Gpi1<sup>b</sup>+/Gpi1<sup>c</sup>– chimaeras were crossed to albino Gpi1<sup>c</sup>– mice, as outlined in Fig. 1B. At least three albino first generation (G1) offspring of each chimaera and all pigmented G1 offspring were typed for GPI to check they were all GPI1C. Albino GPI1C, G1 offspring were expected to be Gpi1<sup>c</sup>– homozygotes whereas pigmented GPI1C, G1 offspring were expected to be Gpi1<sup>c</sup>– heterozygotes. To check that all pigmented G1 mice were Gpi1<sup>c</sup>– heterozygotes, they were crossed to Gpi1<sup>b</sup>b mice to produce G2 offspring. G2 mice were typed for GPI to check there were approximately equal numbers of GPI1B (Gpi1<sup>b</sup>b) and GPI1BC (Gpi1<sup>b</sup>b) individuals (Fig. 1B).

**Statistics**

The choice of parametric or non-parametric tests was guided, in part, by normality tests. GraphPad Prism 5.0c (GraphPad Software, Inc. San Diego, CA) was used for most statistical tests, as described in the text. An online statistical calculator (http://vassarstats.net/index.html) was used for chi square goodness-of-fit tests.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

M.A.K. and J.H.F. performed the experiments, J.D.W. and M.A.K. analysed the data and prepared the figures, J.D.W. designed the experiment, supervised the work and wrote the first draft. All authors contributed to the preparation of the final manuscript.

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