ABSTRACT

In any given species, the lifespan of a red blood cell (RBC) is a well-defined and regulated process. However, the mechanism(s) by which the reticuloendothelial system distinguishes when to clear an RBC from circulation are not fully understood. Basic understanding of RBC senescence and clearance relates to a number of pathologies: including primary disorders of RBC lifespan and additional pathologies that affect RBCs (*e.g.* malaria infection, hemolytic toxicologies, G6PD deficiency, etc.). An additional issue that is highly related to the basic question of how RBCs are cleared, but more focused on translational biology of a therapeutic product, is the issue of clearance of transfused RBCs after storage. The average bag of blood has 25% of the intact transfused RBCs clear from circulation within the first 24 hours after transfusion. Thus, for a patient who receives 4 units of blood, the equivalent of an entire unit of RBCs is cleared by the reticuloendothelial system. Recent animal data indicate that these rapidly cleared RBCs are potentially toxic to the recipient. Approximately 15,000,000 units of RBCs are transfused each year (in the USA alone) into an estimated 5,000,000 recipients (~1/60 Americans annually). Despite lacking a nucleus, mitochondria, or ribosomes, RBCs nevertheless have mechanisms of programmed cell death that are conceptually similar to apoptosis, but occur by biologically distinct mechanisms (called eryptosis). It is unclear from current data the extent to which eryptosis plays a role in normal RBC senescence. Moreover, as RBC storage is a distinct unnatural insult, it has been hypothesized that eryptosis may play a central role in the "RBC storage lesion". In this context, the current grant application utilizes *in vivo* murine methods of testing RBC senescence and a murine model systems of RBC storage (developed in our lab) to test distinct mechanistic hypotheses. In addition, well-defined murine genetics are utilized to pursue unique discovery. We proposed the following specific aims.

Specific Aim 1: Investigate the Role of Eryptosis in RBC Senescence and Storage Biology: Hypotheses will be tested regarding the role of known eryptotic pathways and effector molecules.

Specific Aim 2: Genetic Determinants of RBC Storage Biology: Well defined strain differences, mating strategies, genomic analysis, and phenotypic characterization will be utilized to perform a quantitative trait loci study to isolate novel genetic determinants associated with normal RBC clearance and also post-transfusion survival of stored RBCs. In addition, metabolomics data will be generated, allowing correlation of metabolic pathways to RBC storage and isolation of candidate genes affecting relevant metabolism.

Specific Aim 3: Investigate the Causal Role of Candidate Genes in RBC Storage Biology: Genetically modified animals will be used to test discrete hypotheses regarding causal and mechanistic roles of gene products in normal clearance of senescent RBCs and also post-transfusion clearance of stored RBCs. This approach will be applied to both new candidate genes and already identified specific gene products.

Specific Aims: In any given species, the lifespan of a red blood cell (RBC) is a well-defined and regulated process. For example, in humans, the average lifespan of an RBC is 120 days. Of course, as with most phenotypes, there is variation between members of a species; nevertheless, RBC lifespan is a well regulated and defined process. RBC turnover is amongst the largest regeneration process of any cell type in the mammalian body. The average person recycles 10 cc of packed RBCs per day, resulting in the annual re-synthesis of a volume of cells equivalent to the average liver. As RBCs lack either a nucleus or ribosomes, they do not synthesize new proteins; however, they are metabolically active cells with active signaling processes. It remains a matter of debate in the field as to the mechanism(s) by which RBCs are removed from circulation at the end of their lifespan; in other words, what happens to an RBC that targets it for removal by the reticuloendothelial system. An issue that is highly related to the basic question of how RBCs are cleared, but more focused on translational biology of a therapeutic product, is the issue of clearance of transfused RBCs after storage. The average bag of blood has 25% of the intact transfused RBCs clear from circulation within the first 24 hours after transfusion. Thus, for a patient who receives 4 units of blood, the equivalent of an entire unit of RBCs is cleared by the reticuloendothelial system. Approximately 15,000,000 units of RBCs are transfused each year (in the USA alone) into an estimated 5,000,000 recipients. Thus, approximately 1 out of every 60 Americans is transfused each year.

It is unclear if the damage done to RBCs during storage (collectively referred to as the "storage lesion"), is related to the natural process of *in vivo* RBC senescence; however, such need not be the case. Thus, the basic questions of RBC clearance in the context of normal RBC senescence and RBC storage are each important areas in which the development of knowledge would be both of basic and translational utility. There are several distinct hypotheses, which have given rise to competing lines of thought regarding RBC clearance biology. Moreover, there are data that have been interpreted to support (and in many cases refute) each of the hypotheses, both in the context of normal RBC clearance and RBC Storage. Such theories include (but are not limited to): **1)** exposure of senescent antigen on older RBCs resulting in opsonization and clearance by natural autoantibodies to the senescent antigen[1,2], **2)** loss of sialic acid resulting in clearance by lectins on macrophages[3], **3)** induction of eryptosis (an RBC version of apoptosis) resulting in disintegration of RBCs into microparticles that are rapidly cleared due to surface phosphatidylserine (PS) exposure [4,5], **4)** surface exposure of PS on intact whole RBCs[3], **5)** loss of CD47 that normally serves as a "don't eat me" signal, and **6)** loss of cytoskeletal flexibility preventing the RBC from squeezing through splenic architecture[3]. Of course, such hypotheses are not mutually exclusive; in other words, loss of sialic acid or eryptosis may give rise to the senescent antigen recognized by autoantibodies. Likewise, oxidative stress (a popular theory of RBC aging) may give rise to both cytoskeletal rigidity and senescent antigen (*e.g*. through promoting band 3 clustering and hemoglobin adducts).It is worth noting that the majority of the data testing the above hypotheses have been performed *in vitro and/*or with artificial accelerated aging techniques. Of course, such maneuvers in no way invalidate the observations made, but they do require follow up *in vitro* studies. In addition, the theories of clearance are largely endpoint mechanisms of clearance, and in some cases lack more proximal mechanistic understanding. In this context, the current grant application utilizes methods of testing RBC senescence *in vivo* (in mice) and a murine model systems of RBC storage (developed in our lab) to take advantage of genetically altered mice available to test distinct mechanistic hypotheses. In addition, well-defined murine genetics are utilized to pursue unique discovery. In this context, we proposed the following specific aims.

Specific Aim 1: Investigate the Role of Eryptosis in RBC Senescence and Storage Biology: A series of hypotheses will be tested regarding the role of known eryptotic pathways and effector molecules in the process of normal clearance of senescent RBCs and also post-transfusion survival of stored RBCs.

Specific Aim 2: Genetic Determinants of RBC Storage Biology: Well defined strain differences, mating strategies, genomic analysis, and phenotypic characterization will be utilized to perform a quantitative trait loci study in order to isolate novel genetic determinants associated with the biology of normal clearance of senescent RBCs and also post-transfusion survival of stored RBCs. In addition, metabolomics data will be generated, thus allowing both correlation of metabolic pathways to RBC storage, and also focusing on candidate genes tied into particular changes in RBC metabolism.

Specific Aim 3: Investigate the Causal Role of Candidate Genes in RBC Storage Biology: Genetically modified animals will be used to test discrete hypotheses regarding causal and mechanistic roles of gene products in normal clearance of senescent RBCs and also post-transfusion of stored RBCs. This approach will be applied to potential candidate genes identified in aim 2. However, to avoid the success of aim 3 depending upon aim 2, already identified specific gene products will also be tested.

Research Strategy

A. Significance and Background:

Understanding the normal process of RBC senescence and clearance is central to the understanding of pathologies involving altered RBC lifespan. Such diseases may include inborn errors in metabolism or congenital mutations in gene products important to RBC survival. In addition, RBC senescence biology plays a role in acquired RBC disorders, from infection (*e.g.* Malaria), genetic mutation (*e.g.* Thalassemia and G6PD deficiency), and toxicology of ingested/injected substances (*e.g.* polycythemia from testosterone or anemia from certain drugs) [4,5]. An alternate scenario of RBC damage, which is iatrogenic in nature but no less clinically significant, is the damage done to stored RBCs.

In addition to the loss of efficacy of RBCs that don't circulate well after transfusion, in recent years, it has been appreciated that transfused RBC units also have the very real potential to induce serious medical sequelae (see section A2 for clinical data). During RBC storage, a number of distinct chemical entities are generated, and each of these substances have known biologies that lead to distinct hypotheses regarding untoward effects on patients from transfusing stored RBCs. RBC breakdown results in free hemoglobin and microparticles[6], both of which scavenge nitric oxide (NO) and can thus prevent vascular relaxation[6]. In addition, aged RBCs and microparticles have been shown to have procoagulant activity that may increase risk of thrombosis [7-10]. Although mature RBCs don't synthesize proteins, they nevertheless can synthesize eicosanoids (prostaglandins and leukotrienes) through metabolism of arachidonic acid contained in membrane phospholipids [11]. Both prostaglandins and leukotrienes are well known to be potent at inducing a variety of effects, including alteration of vascular tone, vessel leakage, coagulation and immunity. Indeed, the eicosanoids generated in stored human blood are sufficient to prime neutrophils that promote damage in a model of lung injury [11]. In addition, a 24 hour post-transfusion recovery of 75% (current FDA standard for storage) means that 25% of RBCs are cleared within 24 hours of transfusion (mostly by the reticuloendothelial system). We have reported that the RBCs that are cleared in the 24 hours post-transfusion induce a cytokine storm in mice [12,13]. If the mice are primed with endotoxin to model sepsis, transfusion of old (but not fresh) RBCs induces a moribund state [13]. Similar findings have now also been observed in a canine model [14]. Along these lines, advanced glycation endproducts (AGEs) have been observed to form on stored RBCs and there are data to indicate that stored RBCs activate the receptor for advanced glycation endproducts (RAGE), which may lead to endothelial activation and/or inflammation [15,16]. Lastly, it has been known for decades that chronic transfusion leads to iron toxicity in recipients, that requires chelation therapy, and can lead to organ failure and death in extreme cases [17]. More recent studies have shown that large volumes of transfused RBCs can lead to end organ failure and promote growth of ferrofillic pathogens [18,19]. In addition to the above potential and/or known toxicities are the well understood risks of alloimmunization, which in extreme cases of multiply alloimmunized patients, can lead to the inability to find sufficient compatible blood either in a timely fashion or at all. In extreme cases, this leads to substantial morbidity (*i.e.* a patient in sickle crisis waiting days for compatible blood), or in some cases death of patients for whom compatible blood cannot be found. Data in animal models indicates that RBCs become more immunogenic as a result of storage [20]. Finally, in some cases alloimmunization to HLA from transfusion may interfere with organ or bone marrow transplantation.

Given the very large numbers of patients that are exposed to RBC transfusions (1 in every 60 Americans annually), and the presence of both known adverse effects (iron overload and alloimmunization) and the effects for which there are substantial animal data but have not yet been confirmed in humans (free hemoglobin, microparticles, eicosanoids, procoagulant activity, and induction of cytokine storm), understanding the mechanisms, pathology, and pathophysiology of transfusion induced sequelae is of high significance. *Each of these potential toxicities is tied to the biology of RBC storage.* In addition, understanding biology of normal RBC senescence is significant to a wide range of diseases involving altered RBC clearance. The translational significance of the proposed studies is that the understanding gained will serve as the rational basis for development of therapeutics, both regarding pathologies in which altered RBC senescence/clearance is involved and also with regards to RBC storage and transfusion biology.

A1. Donor variation of RBC storage in humans and mice: It has been appreciated for over forty years that there is tremendous variability in how RBCs store from different human donors [21,22]. Even for current blood storage solutions, 24 hour post-transfusion recoveries range from 35% to 100% [22] It has been further observed that RBC storage is reproducible from donation to donation for a given donor [23,24], suggesting a potential genetic component [21,25]. The issue of donor variability has high relevance for several reasons. First, many of the FDA approved storage solutions do not meet the FDA criteria of 75% 24 hour post-transfusion survival after expanding

analysis to broader donor populations than those used for FDA trials. Second, it represents a potential problem to the blood supply, as a certain percentage of RBC units give both poor efficacy, and may also contain increased levels of substances with toxic potential. Third, it indicates underlying differences in human RBC physiology that may be relevant to understanding normal RBC function outside of the context of transfusion.

A2. Clinical data testing the hypothesis that stored RBCs are dangerous. The question of whether or not stored RBCs represent a toxic insult is still in a matter of scientific disagreement. A number of retrospective studies have reported significantly worse medical outcomes in patients who receive RBCs that are stored for longer times (typically 14 days) compared to patients who received younger blood [26-28]. These observations have caused significant concern, with many practitioners now requesting younger blood for their patients. However, additional retrospective studies have found no difference in outcomes between older and younger blood, with some reporting that older blood is safer than younger blood [26-28]. Given the intrinsic problems in retrospective studies, NHLBI has now funded a large scale randomized prospective trial to test the hypothesis that the storage age of blood affects medical outcome in patients with complex cardiac surgery (Red Cell Storage Duration Study (RECESS)) [29]. Additional trials are being carried out by Canadian Blood Services (Age of Blood Evaluation (ABLE) [30], and at the Cleveland Clinic (Red Cell Storage Duration and Outcomes in Cardiac Surgery) . A recent trial was completed in premature infants (Age of Red Blood Cells in Premature Infants (ARIPI)), and showed no difference in patient outcome [31]. However, for the ARIPI trial the "older blood" and the "younger blood" groups had an average storage time only 14.6 and 5.1 days, respectively. Since the poor medical outcomes have been most strongly associated with 14 day or older blood, it is unclear if ARIPI tested the hypothesis. Nevertheless, it is a fair statement that within the current standard of practice in the ARIPI pediatric population, age of blood is not correlated with worse medical outcome. Thus, other than known risks of iron overload and alloimmunization, it remains undetermined if stored RBCs result in a worse clinical outcome than do fresh RBCs. However, what is not controversial is that blood storage consistently results in the generation of a variety of cellular and chemical entities with known biologies of significance in different pathologies (see section A above). What is also not controversial is that there is large variation in the biology and storage from donor to donor. The current proposal investigates genetic and biological mechanisms of RBC biology (and potential toxicology) both in the context of normal RBC senescence and RBC storage.

B. Innovation: We have just recently reported that, as with humans, genetically distinct murine donors vary widely in the storage biology of their RBCs including *in vivo* RBC lifespan, post-transfusion survival of stored RBCs, and RBC metabolomics (both at baseline and during storage) [33]. The proposed studies combine the newly evolving field of metabolomics with standard (but well established) breeding strategies and genomic analysis to allow isolation of genetic elements that correlate with RBC *in vivo* lifespan and storage biology. Finally, we introduce a new murine model of G6PD deficiency with advantages over existing systems (see aim 3). The innovation of this proposal lies in our novel observations, use of refined models and methods (including new mice – see aim 3), and application of advanced technologies to the biological question of RBC senescence *in vivo* and during blood storage.

C. Approach:

C1. Preliminary Studies:

C1.1. Generation of a mouse model of RBC storage and transfusion: We have reported a novel system to model RBC storage in mice [32]. To allow tracking of RBCs after transfusion, but still avoid manipulating and/or damaging cells through chemical labeling, we utilized mice that express GFP in their RBCs as donors and wild-type animals as recipients. We observed that murine RBCs have storage characteristics that are similar to human RBC storage, except that they reach a 75% post-transfusion survival faster than human RBCs (14 days for B6 mice compared to 42 days for humans). However, given that murine RBCs have a normal lifespan of 40-50 days compared to 120 days for human RBCs, 14 days storage is proportionately appropriate.

Mouse RBCs undergo similar morphological and cell surface changes (phosphatidylserine and CD47 levels) as do human RBCs during storage [32]. In addition, metabolic changes are highly similar with regards to multiple biochemical and pathways, including loss of 2,3 –DPG, ongoing glycolysis and generation of lactate, pentose phosphate shunt activity, synthesis of glutathione (GSH), generation of GSSG through anti-oxidant activity and reduction back to GSH [33]. Although no model is perfect (and some differences from humans are observed)[33], the above analysis has demonstrated that we have engineered a murine model of RBC storage and post-storage transfusion, which reflects much of the biology of human RBC storage.

C1.2. Refinement of mouse model of RBC storage. After screening a panel of inbred strains, we identified B6 mice as the strain with the best storing RBCs (based upon post-transfusion recoveries) and FVB mice as the worst storing strain [33]. Using this strain combination, we then refined our model to avoid crossing alloimmune barriers, to avoid expression of GFP in donor RBCs, and also to allow measurement of rapid early clearance (to control for hemolysis during storage) [See Figure 1 for approach and gating strategy]. B6 x FVB F1 mice expressing GFP were used as recipients (called F1-GFP hereafter). By using F1-GFP recipients, neither the B6 nor the FVB donor strain crosses an allobarrier when transfusing RBCs. Moreover, as the donor population is tracked using the GFP-negative gate, donor RBCs are not altered through GFP expression. Finally, a tracer population of fresh HOD RBCs is added to the test RBCs prior to transfusion (HOD mice express a chimeric model antigen on RBCs for which we have monoclonal antibodies)[34]. Both a pretransfusion and all post-transfusion specimens were stained with anti-HOD and analyzed by flow cytometry, allowing a ratio of HOD RBCs to test RBCs. The HOD RBCs were always used fresh (right out of the donor) and are well characterized to have 100% survival in this state[34]. Thus, by calculating the survival of the stored test RBCs to HOD RBCs and normalizing to the pretransfusion ration, we are able to essentially track survival of intact RBCs and control for baseline differences in hemolysis and/or hematocrit in stored RBC units. We have observed (as in our previous findings) that after storage, B6 RBCs survive post-transfusion to a significantly greater extent than do FVB RBCs (Figure 2B), whereas no significant difference is seen using freshly isolated RBCs (Figure 2A).

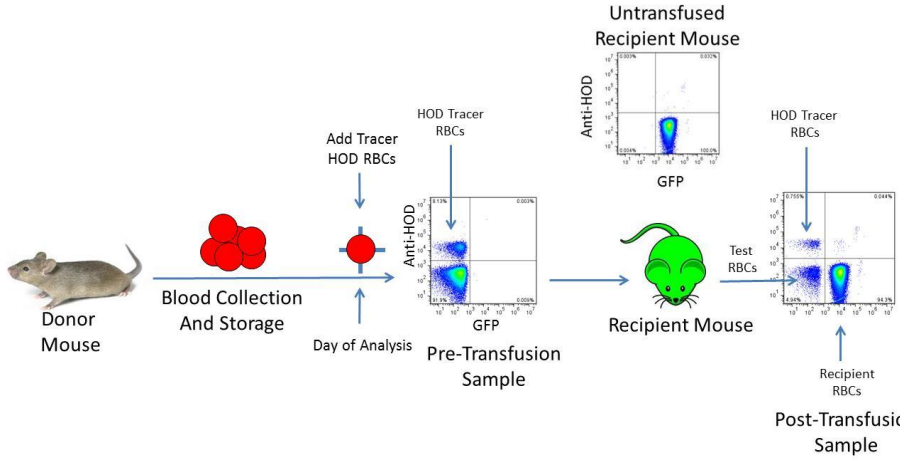

Figure 1 Experimental Design for RBC Storage and Post-Transfusion Recovery

The differences in RBC storage are reflected by a difference in the intrinsic RBC lifespan in the mouse. By biotinylating whole animals and tracing the disappearance of biotin+ RBCs over 50 days, we observed that B6 RBCs appear to have a longer lifespan *in vivo* than do FVB RBCs (Figure 3C). Thus, a juxtaposition of B6 and FVB RBCs serves as a platform to investigate biology of both factors involved in normal RBC lifespan and/or clearance, and also RBC storage biology.

C1.3. Metabolomic Juxtaposition of B6 and FVB RBCs. We have now performed detailed metabolomics analysis of B6 and FVB RBCs, both at time of collection and over the storage process. Metabolomics is essentially a mass spectrometry based quantification of small chemical molecules, and then assignment of those molecules to known metabolic pathways, such that a picture emerges of the cellular biology from a metabolic standpoint. Space limitations prevent presentation of the detailed metabolomics data here, as we have analyzed over 350 different chemical species; however, some main points include the observation of: **1)** lipid peroxidation in FVB RBCs but not B6 RBCs (including generation of 9,10-epoxystearate, 7-α-hydroxycholesterol and 7-β-hydroxycholesterol) [33], **2)** Decreased anti-oxidant pathways in FVB compared to B6 mice, including decreased glutathione (GSH) and cysteine glutathione disulfides, decreased alpha-tocopherol (vitamin E) and decreased ergothioneine

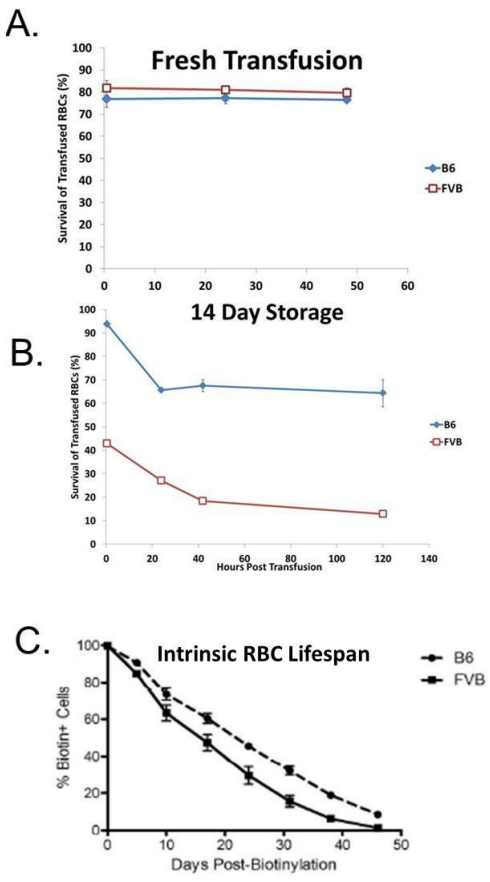

Figure 2: (A) post-transfusion survival of fresh RBCs from B6 or FVB Mice, **(B)** post-transfusion survival of stored RBCs from B6 or FVB mice, **(C)** in vivo lifespan of RBCs as determined by whole animal biotinylation followed by enumerating avidin reactive RBCs.

[33], **3)** indications of decreased glutathione synthesis in FVB compared to B6 mice [33], **4)** highly similar glycolysis, pentose-phosphate shunt, and nucleic acid biology, with a few notable exceptions, including pyruvate, GMP, xanthosine, and urate, and [33] **5)** persistent elevation of cytidine and cytidine metabolites (N4-acetylcytidine) in FVB but not B6 RBCs [33]. Due to space limitations, the interpretation of these findings, the data supporting our conclusions, and additional details can be found in our published work on these observations [33]. Of note is that a number of these differences are present at time point zero (*e.g.* time of collection), and are thus relevant to baseline RBC biology that may reflect differences in RBC senescence *in vivo*.

An additional finding of interest to the issue of untoward effects of blood transfusion (see section A), is that we also observed dramatic differences in the generation of eicosanoids between B6 and FVB RBC units. It has been reported that human RBC units do generate eicosanoids [11], and there are substantial differences from donor to donor (Chris Silliman, personal communication). Both prostaglandin E2 and also the leukotriene precursors (5-HETE, 12-HETE, and 15-HETE) were elevated as a function of time for FVB RBC storage but not B6 RBC storage. The same pattern was seen for HODE species, which are linoleic acid metabolites (we did not resolve 13-HODE from 9-HODE). Presumably, the prostaglandins and leukotrienes were the result of cyclo-oxygenase and 5-lipoxygenase activity on arachidonic acid, respectively. Although arachidonic acid levels were slightly higher at day 0 in FVB than B6, later time points had very similar levels. This can be explained by the rapid generation of lysophospholipids (1-palmitoylglycerophospholnositol and 1-stearoylglycerophosphoinositol) in FVB but not B6. These lysophospholipids are the byproduct of cleavage of arachidonic acid from the sn-2 position of phospholipids, indicating that FVB is likely producing substantial arachidonic acid that is being rapidly converted into eicosanoids. This may be partly explained by the known deficiency in B6 mice of a secreted phospholipase A2; however, this would not explain the lack of conversion of arachidonic acid into eicosanoids in B6 mice. Of note, neither cellular nor secreted phospholipase A2 has been detected in human RBC units. Rather, peroxiredoxin-6 has been implicated in eicosanoid generation in human RBC units [35,36].

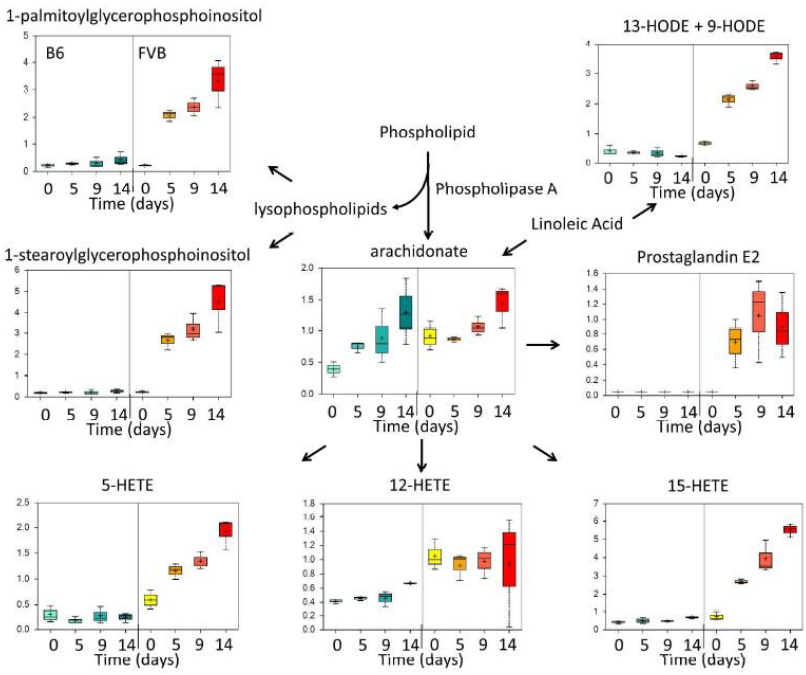

Figure 3 Metabolomics of Eicosanoid Generation During Storage of RBCs from B6 or FVB mice. *Chemicals are in relative amounts based upon ratios of integrated peaks, and thus do not have specific units attached.*

C1.4 Modification of RBC Storage for Single Donors and Initial Characterization of F2 Generation: In order to better understand the genetics of RBC storage properties for B6 vs. FVB mice, we bred B6xFVB mice and found that the F1 animals had intermediate clearance (data not shown due to space limitations). We then interbred the F1 mice to generate an F1xF1 generation of F2 mice. As each mouse is expected to be genetically distinct, due to both random segregation and recombination of chromosomes, pooling blood from multiple donors is not informative. Rather, the assay system was modified to test a single animal at a time. This was accomplished by two separate methods. Terminal bleeds are performed by sterile cardiac puncture, drawing a fixed volume from each donor (500 microliters). Alternatively, a single full capillary tube of blood is collected by retro-orbital bleeding, allowing a standardized volume based upon the capillary tube (this allows the donor to survive to allow subsequent breeding). In either case the blood is combined with a volume adjusted amount of CPDA-1 (as per normal RBC storage) and stored at a 75% hematocrit.

Both the terminal and survival approaches gave similar results on multiple B6 and FVB donors (data not shown). This analysis was then extended to individual F2 mice. An example of 24 hour post-transfusion survivals from

an initial panel of 24 F2 mice is shown (Figure 4A). For mice that had survival bleeding, repeat studies on the same animals demonstrated that the storage phenotype was consistent over time (data not shown). SNP analysis was performed on each of the F2 mice in figure 4A (by [redacted], co-investigator) and QTL analysis was applied to this pilot cohort (by Dr. [redacted], co-investigator). A clear signal was seen on Chromosome 1, with a number of SNPs showing strong association with RBC storage (strongest SNP had $P=10^{-9}$). Additional correlations are observed on chromosomes 4, 9, 12, and 13, none of which achieve statistical significance in this pilot study. In a definitive study with a large sample size of mice (i.e. more opportunities for recombination), finer resolution would be achieved on the signal on Chromosome 1, to allow assessments of particular genes. In addition, correlations on Chromosomes 4, 9, 12, and 13 would be further evaluated, and if significant, the peak regions would be more finely mapped.

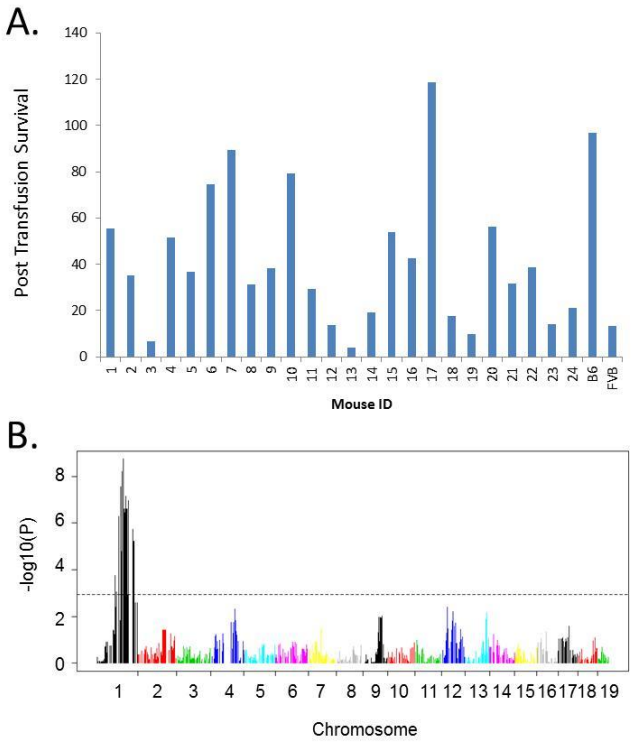

Figure 4: (A) Range of post-transfusion survival of RBCs (stored for 7 days) from different F2 Mice. **(B)** Manhattan plot of initial QTL analysis on the 24 mice shown in A. Horizontal line corresponds to an FDR of q=0.01, which corresponds to P=0.0012

C2. Experimental Approaches:

C3 Specific Aim 1: Investigate the Role of Eryptosis in RBC Senescence and Storage Biology

C3.1. Rational: Current thinking makes a strong distinction between eryptosis and "normal RBC senescence". Senescence is typically seen as a pre-programed lifespan wherein an RBC will undergo changes that result in its removal after it lives to a certain average life-span. In contrast, eryptosis is seen as a safe way to dispose of damaged RBCs that need to be removed before their normal senescent time (akin to apoptosis being safer than necrosis for nucleated cells). However, the possibility remains that eryptosis may play an important role in normal RBC senescence; indeed a number of mouse models have demonstrated genetic deficiencies that enhance or reduce eryptosis and affect RBC lifespan [4,5].

A second issue regards the role that eryptosis plays in the RBC storage lesion. RBC storage certainly constitutes an unnatural condition for RBCs that results in a distinct insult. To the best of our knowledge, the role of eryptosis in RBC aging during storage has not been thoroughly investigated. However, if eryptosis was occurring during RBC storage, then one would expect to see a substantial decrease in RBCs with normal size, a dramatic increase in cells with PS on the surface, and a generation of small debris from the eryptotic process. To the contrary, both data from human and mouse RBC storage indicate that in leukoreduced units of RBCs, which have 25% post-transfusion clearance of RBCs at 24 hours, none of these classic eryptotic changes are seen [32,37]. Thus, one might argue that eryptosis doesn't occur during RBC storage. However, an essential consideration is that RBC storage solutions uniformly contain high concentrations of citrate, to chelate calcium and prevent clotting. As influx of calcium is necessary for eryptosis to proceed, we speculate that eryptosis is halted during RBC storage, but may resume upon exposure to calcium (*e.g.* after transfusion). Indeed, we have observed that incubating stored RBCs with calcium results in substantial RBC fragmentation, which doesn't occur with fresh RBCs (data not shown to space limitations).

C3.2. Hypotheses: Eryptosis plays a role in the lifespan of normal RBCs and is also responsible for the rapid clearance phase observed after transfusion of stored RBCs.

C3.3. Experimental Approaches: The literature on mechanisms or eryptosis is complicated, with different initiators of eryptosis resulting in variations on the downstream effector mechanism of RBC destruction. However, the pathways terminate in common effector molecules that destroy the RBCs, including calpains (cysteine endopeptidases that degrade cytoskeletal proteins and lead to blebbing) and transglutaminase 2 (TG2), which crosslinks proteins resulting RBC rigidity and loss of rheological properties [4].

Gene Knockout Studies

Knocking Calpain Out of RBCs. RBCs express the ubiquitous caplains (μ-calpain and m-calpain). Capn4 is a common regulatory subunit, which forms a heterodimer with capn1 (to make (μ-calpain) or capn2 (to make m-calpain). Targeted deletion of Capn4 eliminates both μ-calpain and m-calpain activities, and is an embryonic lethal deletion. To circumvent this limitation, Tan *et al.* generated a loxP/cre based conditional knockout of Capn4 [38]. This mouse has been generously shared with us by Dr. [redacted], and we have now crossed this mouse with a mouse that expresses CRE in an RBC specific fashion (a generous gift from Dr. [redacted]) [39]. We have succeeded in breeding mice that are homozygous for the floxed Capn4 and express RBC specific CRE, giving rise to a predicted conditional knockout of Caplain activity in RBCs. These mice are viable and also fertile, although litter size is somewhat decreased. Capn4 floxed mice without CRE and mice with only CRE (not floxed) serve as negative controls. We are in the process of phenotyping the putative conditional knockout.

Knocking TG2 out of RBCs. Targeted deletion of TG2 results in a significant decrease in macrophage consumption of RBCs in response to calcium and ionomycin [40]. Likewise, the rate of PS exposure was decreased on RBCs from TG2 KO mice compared to wild-type RBCs. Importantly, both the large and small subunits of μ-calpain are substrates for TG2 crosslinking, which results in increased calpain activity [40]. Thus, in addition to crosslinking structural proteins, TG2 also contributes to (but is not required for) Calpain activity [40]. Further analysis of the TG2 KO mice lend support to the view that eryptosis is not central to normal RBC senescence, as despite defects in induction of eryptosis *in vitro*, TG2 KO RBCs did not have an intrinsically longer lifespan than wild-type RBCs [40]. However, these studies do not address the issue of what role TG2 may play in degradation of stored RBC that are then transfused and exposure to calcium. We have acquired the TG2 KO mice, which are healthy and viable, and now have a colony of animals breeding, which have both the predicted genotype (PCR) and phenotype (Western Blot) in which no TG2 is detectable (data not shown).

Experimental Procedures: RBCs from RBC/Capn4 or TG2 KO mice will be collected and stored as per our normal protocol. After 14 days of storage, the RBCs will be transfused into GFP recipients, and survival will be monitored by following the GFP negative population. As with the approach above (see figure 1), a tracer population (HOD RBCs) will be added immediately prior to transfusion and survival will be calculated as a ratio of test RBC/HOD RBCs. The ratio will then be normalized to the pretransfusion test RBC/HOD ratio, so as to allow an enumeration of intact RBCs that are cleared.

Stored units will also be assayed for eryptosis in the stored unit itself (prior to transfusion), through flow cytometric staining with anti-TER119 and analyzing size and staining with Annex V (to test for surface PS). RBCs will also be incubated with Calcium chloride and will be analyzed for both changes in size and also calcium influx (using Fluro 3 : life technologies), which is a standard test for calcium influx with which our lab has experience.

Backcrossing onto different strain backgrounds: We have published, and show in preliminary data that FVB RBCs have both a decreased intrinsic lifespan, and also store quite differently from B6 RBCs, including substantially worse 24 hour post-transfusion recoveries, accumulation of lipid peroxidation[33], and generation of eicosanoids (see figures 2 and 3). Oxidative insults are known to contribute to eryptosis, and eicosanoids have been observed to enhance eryptosis. Thus, we hypothesize that eryptosis plays a role in the worse survival of FVB RBCs, and that FVB RBCs are a model of both *in vivo* RBC lifespan and RBC storage in which eryptosis is involved. We predict that even if eryptosis is ruled out as being required in B6 RBC biology, that it may play a role on the FVB genetic background. Accordingly, both of the above knockout systems will be backcrossed onto an FVB background. In collaboration with our coinvestigator ([redacted]) and the [redacted] core, we will perform speed congenic backcrossing so as to complete the process in 5-6 generations (1-1.5 years). An additional year will be required to interbreed and validate genotype/phenotypes. Starting in year 3 of the grant, the same experiments (as described above for B6) will be carried out on a FVB background. These studies will entail both RBC storage and also *in vivo* lifespan, by the demonstrated biotinylation method (see preliminary data, figure 2C).

Pharmacological Inhibition Studies

Protein kinase CK1 (casein kinase 1) has been reported to be expressed by both human and murine RBCs, and has recently been reported to be required for eryptosis to occur [41]. D4476 is a commercially available inhibitor of casein kinase 1 (Tocris Bioscience, Cat. No 2902), which has been shown to inhibit eryptosis of both human and murine RBCs in response to either glucose deprivation or oxidative stress [41]. D4476 can be given orally to mice with a high bioavailability. Thus, mice will be dosed with D4476 and RBC in vivo lifespan will be determined

by the biotinylation method (see figure 2C). D4476 injections will be maintained over the course of the RBC lifespan determination.

To test RBC storage biology, wild-type B6 or FVB RBCs will be incubated with D4476 during storage. To maintain inhibition after transfusion, recipient GFPxFVB mice will be dosed with D4476 prior to transfusion. Stored RBCs will then be spiked with tracer HOD RBCs (see Fig 1) and RBCs will be transfused. Control conditions will include RBCs stored and transfused in the same fashion, but in the absence of D4476. Additional controls will consist of wild-type RBCs that have been treated with Calcium/Ionomycin in the absence or presence of D4476, to serve as a positive control for eryptosis detection and to confirm that the D4476 is pharmacologically active and inhibiting eryptosis as intended.

C3.4. Predicted Outcome and Potential Problems: We predict that eryptosis will play a role in both *in vivo* lifespan and also in RBCs during storage. In other words, we predict that the lifespan of RBCs (in situ or post-transfusion) will be extended when eryptosis pathways are disrupted. However, the proposed experiments also have the capacity to reject the hypothesis should eryptosis not be involved, and this would be an important pertinent negative to publish. The lack of a change in RBC lifespan in Tgm2 KO mice is relevant to the above hypothesis(es), however, TgM2 is not as central as calpain. Moreover, as FVB RBCs generate more oxidative damage during storage (see figure 3), which is known to induce eryptosis, we predict breeding the TgM KO onto an FVB background will uncover activities that may not be visible in B6 mice. As each of the proposed genetically modified animals is currently breeding productively in our lab and we are currently performing each of the proposed techniques, we do not anticipate any unusual technical difficulties. We do not have experience using D4476, but have sufficient assays and controls already in place that we will be able to assess its inhibitory activity. We have purposefully combined both genetic and inhibitory approaches, given the distinct strengths and weaknesses of each, to allow multiple approaches to the same question. Thus, we predict that regardless of outcome, useful new knowledge will be generated.

It is worth considering what the meaning would be if it was observed that eryptosis played a role in the FVB but not B6 mouse, for either intrinsic RBC lifespan or RBC storage. We acknowledge first and foremost that mice are a model system that may not reflect human biology; which is a risk to all model systems. However, given the inability to carry out such studies in humans, it is our view that this is a valid approach, so long as we remain mindful of potential species differences. Second, seeing an effect on one strain background but not the other strain background (*e.g.* FVB but not B6) is often a source of concern, as it undermines the ability to infer generalized knowledge from a given strain. However, in the current situation, it is well documented that there is wide variety in human RBC biology from donor to donor, likely of a genetic nature [21-25]. Thus, finding strain to strain variability in mice may very well be relevant to a biology that is divergent in the human population.

C4. Specific Aim 2: Genetic Determinants of RBC Storage Biology and Metabolism

Rationale: A distinct advantage of the murine systems being used is complete genomic sequencing of the mouse strains utilized (as per Jackson Labs), advanced genomic tools, simplified genetic complexity (inbred strains), and rapid gestational times and high fecundity. We have refined our methodology to allow RBC storage analysis of a single donor (see section C1.2). Quantitative Trait Locus (QTL) analysis is now a highly refined methodology for identifying candidate regions of DNA that correlated with phenotype [42]. By combining a variety in phenotype(s) with high resolution SNP arrays of inherited elements and performing QTL statistical analysis, candidate genes affecting RBC biology can be isolated.

Primary Hypotheses for aim 2: QTL analysis will identify candidate genes involved in RBC *in vivo* lifespans and RBC biochemical storage phenotypes using a well-defined F2 population.

Experimental Design (see Figure 5): The breeding strategy will be to cross B6xFVB F1 mice to each other so as to generate a large panel of F2 animals (as in Figure 4). Each F2 mouse will be subjected to the following analysis: **1)** 600 microliters of blood will be obtained by a terminal bleed through sterile intra-cardiac puncture. **2)** 30 microliters of the blood will be utilized to obtain a detailed complete blood count (CBC) (using a Hemavet 950 calibrated for murine work (Drew Scientific Group)). **3)** The remaining blood will be centrifuged, “packed”, and stored for 7 days by above methods. **4)** After 7 days of storage, spontaneous hemolysis will be measured using Drabkin’s reagent (Sigma-Aldrich). **5)** 200 microliters of RBCs will be spiked with 10 microliters of HOD tracer RBCs and will be transfused into an F1-GFP mouse to allow survival curves of transfused RBCs to be determined (as in figure 4). The remaining RBCs (approximately 200 microliters) will be snap-frozen using liquid nitrogen and stored. After all of the animals have been harvested (400 mice in total) all 400 frozen blood samples will be subjected to high resolution LC-MS/MS metabolomics analysis in collaboration with Metablon Inc.

(Durham NC). At the time of sacrifice, 1 cm of tail tissue will be frozen and sent to Dr. [redacted] (co-investigator on this grant) for high resolution SNP analysis; an additional cm of tail tissue will be frozen and archived at -80 as both a back-up sample and also for any future unanticipated analysis that may become useful later (*e.g.* sequencing, methylation analysis, etc.).

A separate 200 mice will have their *in vivo* RBC lifespan determined though whole animal biotinylation and weekly analysis of avidin-reactive RBCs (see figure 2C). Tail DNA will likewise be subjected to high resolution SNP analysis from each animal. After biotinylation studies are finished and biotinylated cells have been undetectable for several weeks, mice will be exsanguinated and both CBC data will be collected and blood will be snap frozen. After all samples of collected, the frozen RBCs will be subjected to LC-MS/MS to allow metabolomic analysis of baseline biology.

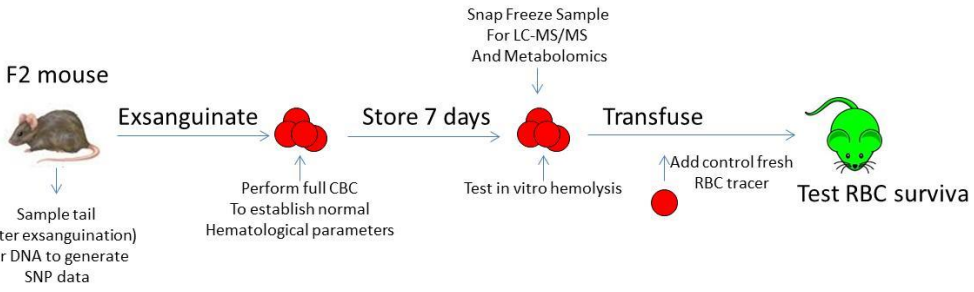

Figure 5 General Experimental Design for F2 Mouse Analysis

Dr. [redacted] (co-investigator on this grant) is a genetics statistician who is an expert in QTL analysis, with multiple recent publications in this field with both complicated genetics and complex binary traits [43-56]. In addition, he has published genetic analyses of hematological traits in mice using similar approaches [53]. In the first phase of analysis Dr. [redacted] will carry out a QTL analysis on the two cohorts of mice, to identify candidate genes linked to the phenotypes of *in vivo* RBC lifespan or post-transfusion recovery of stored RBCs, respectively – an extension of the pilot studies shown in Figure 4. In addition, QTL analysis will also be carried out for baseline hematological parameters (i.e. CBC) and spontaneous (in the unit) hemolysis after storage but prior to transfusion (*e.g*. free measured hemoglobin).

A second phase of analysis will consist of calculating separate correlation coefficients for each measured metabolite (from relative quantitation by LC-MS/MS) with post-transfusion RBC survival, CBC data, spontaneous hemolysis (1st cohort of 400 mice) or for *in vivo* RBC lifespan (2nd cohort of 200 mice). It is predicted that for Metabolites and pathways with strong correlations (negative or positive) with the RBC phenotypes, that QTL analysis will identify the same or similar Loci as the primary QTL for the RBC phenotypes themselves, and as such will serve as a secondary validation of the first phase QTL analysis.

A third phase will consist of QTL analysis of metabolites of interest, which do not correlate with the primary RBC phenotypes. Although the metabolites that fall in this category will not be clear until after full metabolomics data is obtained, one example is the generation of eicosanoids. As detailed above (see section A), the accumulation of eicosanoids in RBC units is of primary interest, as this class of molecules has been implicated in TRALI and also is known to have substantial biological effects in other pathologies and pathophysiologies that may be affected by transfusion. However, it is unclear if the phenotype of eicosanoid generation and poor RBC storage (both seen in FVB mice – see sections C1.2-C1.3) will be related. In the case they are not, the candidate genes that regulate eicosanoid generation in RBC units are themselves of primary interest, whether or not a correlation to primary RBC phenotype is observed.

Statistical Analysis and Determinations: In collaboration with [redacted] (co-investigator on this grant), we have analyzed the post-transfusion RBC survival of 24 different F2 mice (see figure 4). We tested the hypothesis that the phenotype distribution is Gaussian by subjecting the data to a One-Sample Kolmogorov-Smirnov test, which indicated that this phenotype is Gaussian and has a normal distribution. Based upon these initial data, sample size consideration was carried out by a power analysis. It was estimated that 400 mice would result in an ability to detect as low as a 6.5% difference in phenotypic means at 90% power. 200 mice would allow a minimum effect size of 9.0% at 90% power. Coefficients of variation of the assays were taken into consideration based upon repeat measures of the parameters (statistical analyses not shown due to space limitations). Based upon considerations of cost, feasibility, and statistical power, 400 mice are estimated as the optimal number for RBC storage and 200 mice as the number for *in vivo* lifespan determinations.

Predicted Outcome and Potential Problems: We predict that after collecting all of the phenotypic and genetic data, that QTL analysis will identify small regions of the genome (containing a limited number of candidate genes)

that correlate strongly with the primary RBC phenotypes and also selected metabolites. Testing *in vivo* RBC lifespan, measurement of baseline hematological parameters, measurement of spontaneous hemolysis during RBC storage, and determination of post-transfusion RBC survival are each up and running in Dr. [redacted]'s lab at the [redacted] is. Dr. [redacted] is director of the core facility at [redacted], with extensive experience of DNA isolation and SNP analysis in B6 and FVB mice, and high resolution data has been generated with this group in pilot studies for this grant. LC-MS/MS will be carried out by Metabolon Inc., which is a world leader in generating metabolomics data, and with whom Dr. [redacted] has already successfully carried out several projects, including a peer-review publication and generating primary data for this grant (see section C1.3) [33]. Dr. [redacted] is a genetics statistician, a leader in the field of QTL statistics and analysis, and will carry out the QTL and correlation analysis for the generated data. Thus, while the described study is sizable, it is not anticipated that there will be any unusual technical difficulties in performing the proposed studies. We have considered the potential of using the same mice for *in vivo* determination, followed by RBC storage studies (*i.e*. not two separate cohorts). However, to be safe and avoid risking that biotinylation may change the underlying RBC storage biology (even after biotin has cleared) we are keeping these mice as separate groups. One potential problem is that RBCs of the individual F2 mice are stored without leukoreduction, as a necessity, as the blood from a single donor is too little to allow filter leukoreduction. However, in collaboration with Dr. [redacted], we have demonstrated that non-leukoreduced RBCs show the same pattern of eicosanoid generation as leukoreduced RBCs (as in figure 3 – data not shown due to space limits). Thus, while there may be certain differences from contributions of leukocytes, major pathways of interest are not altered. Moreover, as much of the blood in the USA is not leukoreduced, whatever changes are introduced are still of clinical interest.

RBCs from F2 mice will be harvested in batches of 10 donors. In every group of 10 F2 mice, RBCs from several wild-type parental B6 and FVB mice will also be harvested (individually), both as an internal control for validity of any given group of samples, and also for the ability to normalize from lot to lot of collected RBCs. By this approach, harvesting 1-2 lots of 10 F2 mice per week, we anticipate being able to complete the specimen collection in 12 – 18 months (assuming inevitable delays due to breeding coordination). Based upon experience of scientists at Metabolon (personal communication), metabolites found in snap-frozen RBC specimens are stable at -80^0C for at least 2 years. However, as B6 and FVB RBCs will be collected with each batch for F2 mice, this will also serve as a metric for unanticipated lability of any particular metabolite over the time of collection of the F2 RBC mice.

A major limitation to QTL analysis is that one can never proceed past correlation (and to causality) by observation alone. It is for this reason that aim 3 (see below) is specifically designed to test the mechanistic role of genetic elements that score highly on QTL analysis, through interventional experimentation.

Despite the many similarities between mice and humans, there are also many distinct differences, and thus any studies carried out in a murine model suffer the possibility that discoveries will not translate into the human setting. On the surface, this is particularly the case for SNPs, since even though there are many orthologues between humans and mice (with varying degrees of amino acid identity), even if a gene product was involved in both human and murine RBC storage, it is almost unthinkable that a particular SNP (in either a coding or non-coding region), would correlate between humans and mice. Indeed, this is neither a prediction nor an expectation of the investigators on this grant. However, the vast majority of the base metabolic pathways do line up in humans and mice[33]. Thus, it is our prediction that while a given SNP will almost certainly not correlate, and that a particular gene product (or regulator region) may very well also not correlate, that the enzymatic pathways or analogous structural groups of proteins have a much higher likelihood of lining up between species. Thus, identification of an enzyme in a given metabolic pathway or in a general structural feature of RBCs in mice would lead to a search for differences in members of that same pathway or analogous structures in human RBCs (outside the scope of the current proposal).

Finally, the applicants would like to acknowledge up front that this is a relatively high risk aim (euphemistically referred to as "hypothesis generating" and despairingly referred to as "a fishing expedition"). However, it is our position that the phenotypes are sufficiently defined, the genetics of the relevant mouse strains sufficiently resolved, and the QTL approach sufficiently validated by our pilot data (see Figure 4), that there is a high likelihood of the generation of new knowledge that is meaningful, of potential impact, and susceptible to further rigorous mechanistic testing (see aim 3).

C5. Specific Aim 3: Investigate the Causal Role of Candidate Genes in RBC Lifespan and Storage Biology

Overall Rationale: As detailed above, while inbreeding strategies and QTL analysis are powerful tools to isolate candidate genes, one cannot proceed past correlation with such observational tools. In order to test causality, one must have the ability to selectively alter an isolated variable and observe the outcome. This aim is designed to provide interventional studies to test distinct mechanistic hypotheses, divided into two subaims.

C5.3 SUBAIM 1 –Experiments to Assess Causal Role of Genetic Elements Isolated in Aim 2

Rationale: Candidate genetic elements identified in aim 2 will be further refined based upon known expression in RBCs (the RBC proteome), or to structural features that would predict likely involvement. It is also essential to maintain an open mind, as erythropoiesis can be affected by non-erythroid tissues and stored RBCs are bathed in plasma. Accordingly, genetic determinants that encode products found outside of RBCs themselves may have profound influence on RBC biology. It is impossible to predict what SNPs will be identified by QTL analysis, however it is reasonable to predict that SNPs will be identified. In this subaim, the SNPs judged to be most significant (both statistically and based upon predicted biology) will be backcrossed onto the opposite parental strain from which they were derived. This will allow the isolation of an independent variable.

Hypothesis: Genetic elements close to identified SNPs will have causal roles in measured phenotypes of RBCs.

Experimental Design: All SNP(s) identified by QTL analysis in aim 1 will be ranked based upon statistical significance of correlation to phenotype and predicted biology. A SNP itself may be followed, but unless it is hypothesized to be a "causative SNP", it will be close to a polymorphism (or difference) that is the actual candidate. Genetic elements close to the SNP will be evaluated to look for mutations in coding regions of genes, untranslated regions of predicted mRNAs and predicted regulatory regions. Potential for variation in microRNAs will also be evaluated. The suspect genetic difference will then be backcrossed onto the opposite parental strain (B6 or FVB) from which it is derived. As an example, a SNP associated with poor storage and derived from FVB would be backcrossed onto B6 mice. This could also be associated with any particular metabolomic signature of interest (correlating or not to a given hematological parameter). High resolution SNP arrays (to eliminate other donor strain genetic elements) will allow backcrossing to be completed after 4-5 generations instead of the traditional 9-10 generations. Moreover, higher resolution tests may be generated to backcrossed mice with recombinations closer to the candidate genetic difference. After backcrossing is complete, the resultant "congenic strains" will be either tested directly or intercrossed to return the candidate genetic element to homozygosity (as may be needed for recessive traits). As multiple animals will be available (due to generating a new line) donor mice will not be in limiting supply. The phenotypes of the resultant animals will be fully tested, including: 1) *in vivo* RBC lifespan, 2) hematology parameters, 2) RBC survival after storage, 3) spontaneous hemolysis, and 4) metabolic profile during storage.

As a second approach, concurrent breeding will be carried out, using our system of survival donation of RBCs (see preliminary data). F2 mice that have RBCs that store very well or very poorly will be backcrossed to the opposite parental strain (FVB or B6, respectively). If the offspring maintain the altered phenotype, then backcrossing will continue. If not, then the mice will be interbred to return traits to homozygosity in 25% of the animals, and phenotype will be re-evaluated. Backcrossing will continue 5-10 generations, and will be followed by high resolution SNP analysis. This approach will use phenotype itself and selective breeding to focus in on genetic elements that determine RBC biology. In this way, the success of the aim is not dependent upon the success of aim 2 and the generation of candidates by QTL analysis.

Predicted outcome and potential problems: We predict that selective backcrossing following a genotype (in this case SNPs or nearby genetic determinants identified by QTL analysis) or following phenotype, will allow us to isolate particular genetic regions as independent variables. The approach serves as a distinct experimental maneuver to test causality for correlations generated by QTL and also to follow phenotype as an alternate approach to QTL. As each of the assays is currently up and running in our lab, we do not anticipate any unusual difficulties. Although new genetic screening tests may need to be generated for novel determinants identified by QTL, such assays are now routine, and will be developed by Dr. [redacted], who is the director of the genotyping core and is an expert in SNP and genetic analysis. This approach is very powerful in rejecting candidate QTL elements, which are observed not to confer phenotype after being isolated. However, one problem is that such elements may be involved in phenotype, but not alone sufficient to cause it. Thus, different congenic strains can be interbred to introduce multiple elements simultaneously. This maneuver will be taken in the event that genetic determinants of high interest show no phenotype.

One potential problem with the current approach is that it presupposes that one can isolate the genetic element of interest from all other genetic elements. In other words one always risks the possibility that another element,

which segregates with the identified element, is actually responsible for the phenotype being observed. This can be monitored by the SNP analysis used to follow the mice. As the complete sequence of both B6 and FVB mice are known (the rational for using only mice from Jackson), one can impute segregating elements and screen them out during backcross. In the unlikely event that a segregating polymorphism is so close to a gene of interest that we cannot breed it out by recombination, we have the experimental option of making a direct knockin mouse, in which the desired genetic element is introduced by itself into the genome. We are not specifically proposing the generation of knockin mice, as it may be overambitious in our view for the current application; however, it represents a next step if there are concerns that cannot be addressed by above breeding strategies.

A final concern is that because Subaim 3.1 will result in testing the mechanistic role of candidate genes not yet identified, which will be generated by the proposed QTL analysis in aim 2, it is not possible to specify which candidate genes will specifically be tested. Accordingly, this aim is somewhat dependent upon the success of a previous aim, which is never an optimal construct for a proposed project. However, the alternate approach of selective backcrossing based upon phenotype mitigates risk in this regard. Moreover, subaim 3.2 below focusses on a discrete genetic determinant that has already been identified as a region of interest in RBC lifespan and storage. Thus, even if aim 3.1 has problems due to unanticipated obstacles in aim 2, which are not overcome through phenotype driven backcrossing, subaim 3.2 will nevertheless provide hypothesis based testing of a specific potential causative genetic determinant of RBC storage.

C5.2 SUBAIM 3.2 – Test the role of G6PD Deficiency in RBC Storage

Rationale: Deficiencies in Glucose-6-phosphate dehydrogenase (G6PD) is a highly common phenotype in humans, which like sickle cell trait, is hypothesized to confer protection from malaria. However, the result of G6PD deficiency is that RBCs cannot generate sufficient NADPH (through the pentose phosphate shunt). As NADPH is a co-factor for glutathione reductase, G6PD deficient RBCs are lacking normal glutathione based anti-oxidant pathways. Accordingly, G6PD RBCs are susceptible to hemolysis from oxidative stress (often present due to infection, drugs, or diet). For example, G6PD deficient patients can under hemolysis if treated with primaquine, dapsone, sulfamethoxazone–trimethoprim (Bactrim), or if foods such as Fava Beans are ingested in sufficient quantity. As oxidant stress is commonly observed in RBC storage, it has been hypothesized that G6PD deficiency is a likely donor characteristic that would lead to poorly storing RBCs [57]. This is of particular importance in patients with sickle cell disease, as the phenotypic matching required to minimize alloimmunization to RBC antigens (e.g. D, Kell, Kidd and Duffy) results in directing a higher frequency of G6PD deficient RBC units to sickle cell patients[58,59]. Currently, the RBC blood supply is not screened for G6PD deficiency.

Hypothesis: G6PD deficiency is a genetic determinant of poor RBC storage.

Experimental Design: A mouse model of G6PD deficiency has been described through random ENU based mutagenesis; however, in our view, this is a poor model of the known human biology [60]. G6PD deletion is embryonic lethal in mice, and has not been observed in humans. The defect in the vast majority of human G6PD deficient people consists of mutation(s) that result in decreased stability of the G6PD enzyme. In essentially all tissues, this is not a defect, as increased and ongoing synthesis compensate. However, in mature RBCs, which do not synthesize protein, the G6PD degrades over time, to undetectable levels in the more severe forms of G6PD deficiency [61]. Thus, G6PD deficiency in humans is essentially a RBC specific deficiency, and there is a continuum of RBCs from low G6PD activity (in younger RBCs) to essentially none (in older RBCs), as a function of RBC age. In contrast, the existing mouse models have decreased (but very stable) G6PD activity in all RBCs, and also in non-RBC tissues. From our point of view, this results in numerous differences from the human phenotype.

We are in the process of generating a murine model of human G6PD deficiency. We are knocking the human G6PD variant (Med -), which results in profound G6PD deficiency in humans, into the mouse G6PD locus. Like humans, the G6PD gene is on the X chromosome in mice. Because global G6PD deficiency is embryonic lethal, we are knocking in a construct in which the human Med– gene is present in an inactive form, with the murine G6PD cDNA expressing upstream (Figure 6B). This will allow homologous recombination without G6PD alteration. The Neomycin cassette will be removed using a flp recombinase breeder (Figure 6C). Then, after a viable strain has been obtained, we will cross it with a CRE recombinase transgenic mouse, which will excise the murine form and result in expression of the human med- G6PD (Figure 6D). The advantage to this approach is that if the human med- G6PD be deleterious to the mouse, then we can cross the animal with an RBC specific CRE strain, so as to have a conditional knockin that only effects RBCs and not other tissues, which should mitigate any lethalities or toxicities of the knockin (we are currently breeding the RBC specific Cre transgenic). Moreover, as there are commercially available mice with inducible Cre constructs, we can also generate mice

that don't become Med – until after they are adults and we induce the CRE. As depicted in the design (see figure 6), we have taken care not to disrupt a micro RNA cassette nearby the G6PD gene, nor disrupt an IκB kinase gene that runs antisense on the same chromosome (not depicted in figure 6).

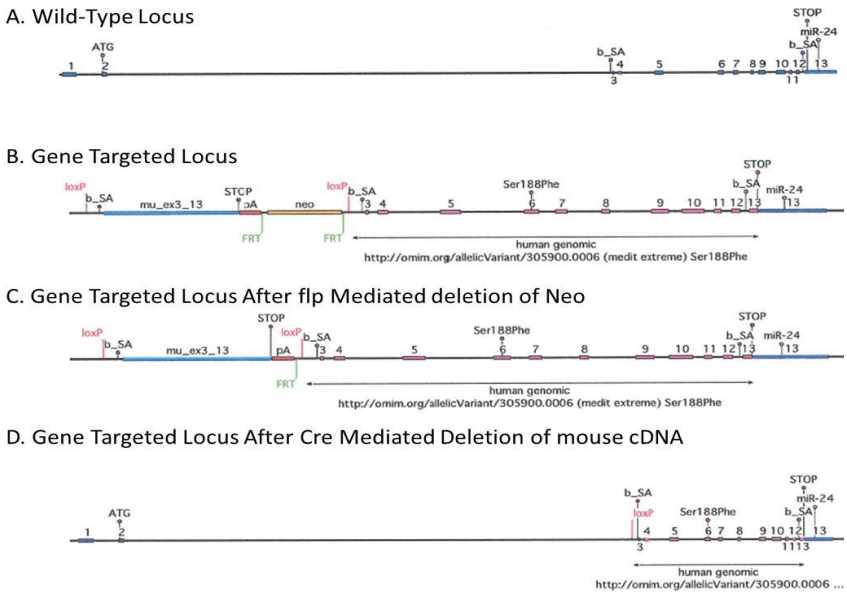

Figure 6 Overall Design of Novel G6PD Deficient Mouse

Once generated, RBCs from this mouse will be tested for their G6PD enzymatic properties, life span *in vivo*, RBC storage properties, and metabolomic properties (including glutathione pathways and oxidative damage) by the approaches and methods described above. This will formally test the role of G6PD deficiency on RBC biology and storage, which is currently a popular hypothesis that lacks sufficient tools to be tested *in vivo*. An additional benefit is that this novel mouse will also serve as a platform for studying related biologies (*e.g.* malaria pathogenesis, drug induced hemolysis, etc.).

Predicted outcome and potential problems: As all of the proposed techniques and assays are currently up and running in our laboratory, we do not anticipate any unusual technical difficulties with the proposed experiments. The knockin mouse is not completed, but is well underway and is being generated with existing funds. Given our success rates in making knockin mice with Ozgene (3 out of 3 projects successfully), and the cautions we have built into the vector, we predict a high likelihood of success. Humans with the Med – mutation are healthy and do not suffer G6PD related illness until they experience an oxidative stress to RBCs; thus we do not anticipate problems in generating the animal. We predict that G6PD deficiency will have a substantial effect upon RBC storage. Of note, we also predict a synergy of observations in the G6PD mouse with other approaches in aim 1. For example, eryptosis is enhanced in human RBCs with G6PD deficiency [62]; thus, mechanistic biology can be investigated by crossing our G6PD deficient mice with RBC calpain null mice or by using D4476, if justified by observations of the G6PD deficient mouse RBC biology.

D. Timeline of Proposed Studies and Team of Investigators Assembled: We anticipate that for aim 1, the B6 based studies will be completed in the first two years, by which time the backcrosses onto FVB should be complete, allowing subsequent FVB analysis in later years. In aim 2, the F2 analysis should be completed by 18 months, with full QTL analysis being completed by 2 years. Likewise, we anticipate that the new "congenic" strains in which phenotypes are backcrossed onto opposite strains would be completed by 2 years. Thus, in the latter 3 years of the grant, the congenic lines will be fully phenotypes, analyzed, and intercrossed if justified. In aim 3, we anticipate the G6PD mouse to be available by the end of year one, and the necessary QTL candidates to be available by the end of year 2, allowing speed backcrossing and analysis as described. We have assembled a diverse group of scientists to allow a multidisciplinary approach to the proposed studies.

Dr. [redacted] is an expert using and generating murine RBC models and RBC storage, and has generated many of the techniques and systems in this grant. Dr. [redacted] is an expert in mouse SNP analysis and genetic backcrossing. Dr. [redacted] is a leading genetic statistician in QTL analysis. Dr. [redacted] is an expert mass spectrometrist, who will perform all the focused mass spec analysis of chosen metabolites after candidates have been isolated from the F2 analysis working with Metabolon Inc. To add expertise and assist in interpretation of phenotypic data from new strains of mice, we have also recruited Dr. [redacted] as an expert on RBC biology and Dr. [redacted] as an expert on RBC storage biology as collaborators (please see letters of support).

Literature Cited:

1. Arese P, Turrini F, Schwarzer E. Band 3/complement-mediated recognition and removal of normally senescent and pathological human erythrocytes. *Cell Physiol Biochem*. 2005;16(4-6):133-146.

2. Lutz HU. Naturally Occurring Autoantibodies in Mediating Clearance of Senescent Red Blood Cells. In: Lutz HU, ed. Naturally Occurring Antibodies (NABs). New York: Landes Bioscience and Springer Science; 2012:76-90.

3. Bratosin D, Mazurier J, Tissier JP, et al. Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages. A review. *Biochimie*. 1998;80(2):173-195.

4. Lang E, Qadri SM, Lang F. Killing me softly - suicidal erythrocyte death. *Int J Biochem Cell Biol*. 2012;44(8):1236-1243.

5. Lang F, Qadri SM. Mechanisms and significance of eryptosis, the suicidal death of erythrocytes. *Blood Purif*. 2012;33(1-3):125-130.

6. Donadee C, Raat NJ, Kanias T, et al. Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. *Circulation*. 2011;124(4):465-476.

7. Cardo LJ, Hmel P, Wilder D. Stored packed red blood cells contain a procoagulant phospholipid reducible by leukodepletion filters and washing. *Transfus Apher Sci*. 2008;38(2):141-147.

8. Gao Y, Lv L, Liu S, Ma G, Su Y. Elevated levels of thrombin-generating microparticles in stored red blood cells. *Vox Sang*. 2013;105(1):11-17.

9. Keating FK, Butenas S, Fung MK, Schneider DJ. Platelet-white blood cell (WBC) interaction, WBC apoptosis, and procoagulant activity in stored red blood cells. *Transfusion*. 2011;51(5):1086-1095.

10. Lu C, Shi J, Yu H, Hou J, Zhou J. Procoagulant activity of long-term stored red blood cells due to phosphatidylserine exposure. *Transfus Med*. 2011;21(3):150-157.

11. Silliman CC, Moore EE, Kelher MR, Khan SY, Gellar L, Elzi DJ. Identification of lipids that accumulate during the routine storage of prestorage leukoreduced red blood cells and cause acute lung injury. *Transfusion*. 2011;51(12):2549-2554.

12. Hod EA, Spitalnik SL. Harmful effects of transfusion of older stored red blood cells: iron and inflammation. *Transfusion*. 2011;51(4):881-885.

13. Hod EA, Zhang N, Sokol SA, et al. Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. *Blood*. 2010;115(21):4284-4292.

14. Callan MB, Patel RT, Rux AH, et al. Transfusion of 28-day-old leucoreduced or non-leucoreduced stored red blood cells induces an inflammatory response in healthy dogs. *Vox Sang*. 2013.

15. Mangalmurti NS, Friedman JL, Wang LC, et al. The receptor for advanced glycation end products mediates lung endothelial activation by RBCs. *Am J Physiol Lung Cell Mol Physiol*. 2013;304(4):L250-263.

16. Mangalmurti NS, Chatterjee S, Cheng G, et al. Advanced glycation end products on stored red blood cells increase endothelial reactive oxygen species generation through interaction with receptor for advanced glycation end products. *Transfusion*. 2010;50(11):2353-2361.

17. Poggiali E, Cassinerio E, Zanaboni L, Cappellini MD. An update on iron chelation therapy. *Blood Transfus*. 2012;10(4):411-422.

18. Baek JH, D'Agnillo F, Vallelian F, et al. Hemoglobin-driven pathophysiology is an in vivo consequence of the red blood cell storage lesion that can be attenuated in guinea pigs by haptoglobin therapy. *J Clin Invest*. 2012;122(4):1444-1458.

19. Solomon SB, Wang D, Sun J, et al. Mortality increases after massive exchange transfusion with older stored blood in canines with experimental pneumonia. *Blood*. 2013;121(9):1663-1672.

20. Hendrickson JE, Hod EA, Spitalnik SL, Hillyer CD, Zimring JC. Storage of murine red blood cells enhances alloantibody responses to an erythroid-specific model antigen. *Transfusion*. 2010;50(3):642-648.

21. Dern RJ, Gwinn RP, Wiorkowski JJ. Studies on the preservation of human blood. I. Variability in erythrocyte storage characteristics among healthy donors. *J Lab Clin Med*. 1966;67(6):955-965.

22. Dumont LJ, AuBuchon JP. Evaluation of proposed FDA criteria for the evaluation of radiolabeled red cell recovery trials. *Transfusion*. 2008;48(6):1053-1060.

23. Hess JR. Scientific problems in the regulation of red blood cell products. *Transfusion*. 2012;52(8):1827-1835.

24. Reid TJ, Babcock JG, Derse-Anthony CP, Hill HR, Lippert LE, Hess JR. The viability of autologous human red cells stored in additive solution 5 and exposed to 25 degrees C for 24 hours. *Transfusion*. 1999;39(9):991-997.

25. Dern RJ, Wiorkowski JJ. Studies on the preservation of human blood. IV. The hereditary component of pre- and poststorage erythrocyte adenosine triphosphate levels. *J Lab Clin Med*. 1969;73(6):1019-1029.

26. Koch CG, Li L, Sessler DI, et al. Duration of red-cell storage and complications after cardiac surgery. *N Engl J Med*. 2008;358(12):1229-1239.

27. Middelburg RA, van de Watering LM, Briet E, van der Bom JG. Storage time of red blood cells and mortality of transfusion recipients. *Transfus Med Rev*. 2013;27(1):36-43.

28. Triulzi DJ, Yazer MH. Clinical studies of the effect of blood storage on patient outcomes. *Transfus Apher Sci*. 2010;43(1):95-106.

29. Steiner ME, Assmann SF, Levy JH, et al. Addressing the question of the effect of RBC storage on clinical outcomes: the Red Cell Storage Duration Study (RECESS) (Section 7). *Transfus Apher Sci*. 2010;43(1):107-116.

30. Lacroix J, Hebert P, Fergusson D, et al. The Age of Blood Evaluation (ABLE) randomized controlled trial: study design. *Transfus Med Rev*. 2011;25(3):197-205.

31. Fergusson DA, Hebert P, Hogan DL, et al. Effect of fresh red blood cell transfusions on clinical outcomes in premature, very low-birth-weight infants: the ARIPI randomized trial. *JAMA*. 2012;308(14):1443-1451.

32. Gilson CR, Kraus TS, Hod EA, et al. A novel mouse model of red blood cell storage and posttransfusion in vivo survival. *Transfusion*. 2009;49(8):1546-1553.

33. Zimring JC, Smith N, Stowell SR, et al. Strain-specific red blood cell storage, metabolism, and eicosanoid generation in a mouse model. *Transfusion*. 2013.

34. Desmarets M, Cadwell CM, Peterson KR, Neades R, Zimring JC. Minor histocompatibility antigens on transfused leukoreduced units of red blood cells induce bone marrow transplant rejection in a mouse model. *Blood*. 2009;114(11):2315-2322.

35. Dzieciatkowska M, Silliman CC, Moore EE, et al. Proteomic analysis of the supernatant of red blood cell units: the effects of storage and leucoreduction. *Vox Sang*. 2013.

36. Silliman CC. Lipids: free fatty acids, eicosanoids, and lysophospholipids and the pro-inflammatory effects of transfusion. . *ASH Meeting 2012 Scientific Program 2012:*. 2012:SCI-48.

37. Bratosin D, Leszczynski S, Sartiaux C, et al. Improved storage of erythrocytes by prior leukodepletion: flow cytometric evaluation of stored erythrocytes. *Cytometry*. 2001;46(6):351-356.

38. Tan Y, Dourdin N, Wu C, De Veyra T, Elce JS, Greer PA. Conditional disruption of ubiquitous calpains in the mouse. *Genesis*. 2006;44(6):297-303.

39. Peterson KR, Fedosyuk H, Zelenchuk L, et al. Transgenic Cre expression mice for generation of erythroid-specific gene alterations. *Genesis*. 2004;39(1):1-9.

40. Sarang Z, Madi A, Koy C, et al. Tissue transglutaminase (TG2) facilitates phosphatidylserine exposure and calpain activity in calcium-induced death of erythrocytes. *Cell Death Differ*. 2007;14(10):1842-1844.

41. Zelenak C, Eberhard M, Jilani K, Qadri SM, Macek B, Lang F. Protein kinase CK1alpha regulates erythrocyte survival. *Cell Physiol Biochem*. 2012;29(1-2):171-180.

42. Mott R, Flint J. Dissecting Quantitative Traits in Mice. *Annu Rev Genomics Hum Genet*. 2013.

43. Jonas E, Thomson PC, Hall EJ, McGill D, Lam MK, Raadsma HW. Mapping quantitative trait loci (QTL) in sheep. IV. Analysis of lactation persistency and extended lactation traits in sheep. *Genet Sel Evol*. 2011;43:22.

44. Hadsell DL, Wei J, Olea W, et al. In silico QTL mapping of maternal nurturing ability with the mouse diversity panel. *Physiol Genomics*. 2012;44(16):787-798.

45. Thomson PC. A generalized estimating equations approach to quantitative trait locus detection of non-normal traits. *Genet Sel Evol*. 2003;35(3):257-280.

46. Cavanagh CR, Jonas E, Hobbs M, Thomson PC, Tammen I, Raadsma HW. Mapping Quantitative Trait Loci (QTL) in sheep. III. QTL for carcass composition traits derived from CT scans and aligned with a meta-assembly for sheep and cattle carcass QTL. *Genet Sel Evol*. 2010;42:36.

47. Miles LG, Isberg SR, Thomson PC, et al. QTL mapping for two commercial traits in farmed saltwater crocodiles (Crocodylus porosus). *Anim Genet*. 2010;41(2):142-149.

48. Raadsma HW, Jonas E, McGill D, Hobbs M, Lam MK, Thomson PC. Mapping quantitative trait loci (QTL) in sheep. II. Meta-assembly and identification of novel QTL for milk production traits in sheep. *Genet Sel Evol*. 2009;41:45.

49. Raadsma HW, Thomson PC, Zenger KR, et al. Mapping quantitative trait loci (QTL) in sheep. I. A new male framework linkage map and QTL for growth rate and body weight. *Genet Sel Evol*. 2009;41:34.

50. Koudande OD, Thomson PC, Bovenhuis H, Iraqi F, Gibson JP, van Arendonk JA. Biphasic survival analysis of trypanotolerance QTL in mice. *Heredity (Edinb)*. 2008;100(4):407-414.

51. Koudande OD, Iraqi F, Thomson PC, Teale AJ, van Arendonk JA. Strategies to optimize marker-assisted introgression of multiple unlinked QTL. *Mamm Genome*. 2000;11(2):145-150.

52. Kirk EP, Hyun C, Thomson PC, et al. Quantitative trait loci modifying cardiac atrial septal morphology and risk of patent foramen ovale in the mouse. *Circ Res*. 2006;98(5):651-658.

53. Cheung CC, Martin IC, Zenger KR, et al. Quantitative trait loci for steady-state platelet count in mice. *Mamm Genome*. 2004;15(10):784-797.

54. Khatkar MS, Thomson PC, Tammen I, Raadsma HW. Quantitative trait loci mapping in dairy cattle: review and meta-analysis. *Genet Sel Evol*. 2004;36(2):163-190.

55. Moradi Marjaneh M, Martin IC, Kirk EP, Harvey RP, Moran C, Thomson PC. QTL mapping of complex binary traits in an advanced intercross line. *Anim Genet*. 2012;43 Suppl 1:97-101.

56. Raadsma HW, Jonas E, Fleet MR, et al. QTL and association analysis for skin and fibre pigmentation in sheep provides evidence of a major causative mutation and epistatic effects. *Anim Genet*. 2013;44(5):547-559.

57. Francis RO, Jhang JS, Pham HP, Hod EA, Zimring JC, Spitalnik SL. Glucose-6-phosphate dehydrogenase deficiency in transfusion medicine: the unknown risks. *Vox Sang*. 2013.

58. Raciti PM, Francis RO, Spitalnik PF, Schwartz J, Jhang JS. Acquired hemoglobin variants and exposure to glucose-6-phosphate dehydrogenase deficient red blood cell units during exchange transfusion for sickle cell disease in a patient requiring antigen-matched blood. *J Clin Apher*. 2013;28(4):325-329.

59. Francis RO, Jhang J, Hendrickson JE, Zimring JC, Hod EA, Spitalnik SL. Frequency of glucose-6-phosphate dehydrogenase-deficient red blood cell units in a metropolitan transfusion service. *Transfusion*. 2013;53(3):606-611.

60. Sanders S, Smith DP, Thomas GA, Williams ED. A glucose-6-phosphate dehydrogenase (G6PD) splice site consensus sequence mutation associated with G6PD enzyme deficiency. *Mutat Res*. 1997;374(1):79-87.

61. Mason PJ, Bautista JM, Gilsanz F. G6PD deficiency: the genotype-phenotype association. *Blood Rev*. 2007;21(5):267-283.

62. Lang KS, Roll B, Myssina S, et al. Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. *Cell Physiol Biochem*. 2002;12:365-372.