ORIGINAL ARTICLES

Transgenic rescue of hemolytic anemia due to red blood cell pyruvate kinase deficiency

Hitoshi Kanno, Taiju Utsugisawa, Shin Aizawa, Tsutomu Koizumi, Ken-ichi Aisaki, Takako Hamada, Hiromi Ogura, Hisaichi Fujii

ABSTRACT

Background and Objectives

Red blood cell pyruvate kinase (R-PK) deficiency is the most common glycolytic enzyme defect associated with hereditary non-spherocytic hemolytic anemia. Cases with the most severe deficiency die in the peri- or neonatal period and no specific therapy exists at present. To test whether the targeted overexpression of the normal R-PK gene in erythroid cells could reduce hemolysis in R-PK mutant mice, we performed a genetic rescue study using human R-PK transgenic mice.

Design and Methods

Human R-PK promoter driven with human μLCR of the human β-globin locus was used for the erythroid-specific expression of human R-PK in murine erythrocytes. The transgenic lines were mated with homozygous R-PK mutant mice and subsequently backcrossed. Mutant homozygotes with the μLCR-R-PK transgene were examined for any therapeutic effects of transgene expression.

Results

Two PK transgenic lines, hRPK_lo and hRPK_hi, were obtained. R-PK activity of the transgenic mice reached as high as three times that of the animals with the endogenous PK gene. Overexpression of human R-PK in the homozygous mutant mice successfully reduced hemolytic anemia. Improvements of hemolysis were evaluated by hemoglobin concentration, reticulocyte count, and spleen weight, which showed significant correlations with the levels of expression of the transgene. Recovery from metabolic disturbance in mutant red blood cells was shown as normalized concentrations of the glycolytic intermediates upstream of PK. In addition, there was a remarkable negative correlation between R-PK activity and the number of TUNEL-positive erythroid progenitors in the spleen.

Interpretation and Conclusions

These results indicate that overexpression of the wild-type PK gene in mutant erythroid cells ameliorates both erythroid apoptosis and the shortened red blood cell lifespan observed in PK mutant mice. It is likely that the level of transgene expression required to achieve evident therapeutic effects should be equivalent to or more than that of the endogenous PK gene. This gene-addition strategy may be suitable for clinical application if there is a high level of transgene expression of R-PK in erythroid progenitors/red blood cells.

Key words: pyruvate kinase deficiency, hemolytic anemia, transgene therapy, RBC enzyme disorders.

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Pyruvate kinase (PK) deficiency is the most common glycolytic enzyme defect associated with hemolytic anemia.[1-3] Although subjects with PK deficiency show a moderate degree of hemolytic anemia, the most severe cases die *in utero*[4,5] or are transfusion-dependent.[6] Repeated red blood cell (RBC) transfusions may induce hemochromatosis,[7] and a recent report showed that free hemoglobin caused by intravascular hemolysis might interfere with the biological action of nitric oxide, leading to the development of pulmonary hypertension.[8]

Hematopoietic stem cells or progenitor cells express the M2-type PK isozyme, while RBC-type PK (R-PK) becomes a major isozyme during erythroid differentiation/maturation.[9,10] In mature RBC, R-PK is the only detectable PK. We recently demonstrated that R-PK is not only important for mature RBC but also anti-apoptotic molecules for erythroid progenitors both in humans[11] and mice.[12] To establish a gene therapy protocol for PK deficiency, the normal R-PK gene should be introduced into hematopoietic stem cells or erythroid progenitor cells, and the transgene must be activated during erythroid differentiation.

We previously identified that hereditary hemolytic anemia spontaneously occurring in an inbred strain of CBA mice was due to PK deficiency.[13] The PK mutant mice show moderate hemolytic anemia and marked splenomegaly. Subsequently, we identified a missense mutation of the murine PK gene,[14] and showed that the mutation substituted the residue Gly338 near the substrate-binding site with Asp. As a result, the mutant PK lost its activity, despite there being almost normal subunit contents in RBC.

Although hematopoietic stem cell transplantation (HSCT) has been considered as a therapeutic strategy for PK deficiency in a PK-deficient subject[15] as well as in animal models,[13,16,17] a curative therapy without any life-threatening complications needs to be developed. In addition, the majority of the identified PK gene mutations are missense mutations,[2,3] and previous studies revealed that there were some kinetically aberrant enzymes, which showed normal intracellular stability in erythroid cells. These results suggest that R-PK subunits derived from the transgene might form heterotetramers with aberrant R-PK. Since heterozygotes of PK gene mutations usually show the normal phenotype, we examined whether a gene-addition strategy is feasible for PK gene therapy. Previously, Tani *et al.* introduced human liver-type PK (L-PK) into murine hematopoietic stem cells and demonstrated the prolonged expression of human L-PK mRNA in both peripheral blood and hematopoietic organs after bone marrow transplantation.[18] In this study, we examined how hemolytic anemia can be phenotypically cured by the genetic rescue of R-PK mutant mice.

Design and Methods

Male and female CBA/N-*PK-1^{slc}/PK-1^{slc}* (hereafter *PK-1^{slc}*), CBA/N-+/+ (hereafter CBA) and C57BL/6 mice, 8-10 weeks of age, were obtained from Japan SLC Harumi Farm (Shizuoka, Japan) and kept under pathogen-free conditions.

We constructed a human β-globin (*HBB*)/human liver and RBC pyruvate kinase (*PKLR*) hybrid gene for the high level expression of human R-PK in erythroid cells (Figure 1). A 1.7-kb *Kpn*I-*Sfi*I genomic DNA fragment covering the 5'-flanking region of the human *PKLR* gene and a 1.7-kb *Sfi*I-StuI human R-PKcDNA fragment were subcloned into *Kpn*I-*Eco*RV sites in pcDNA3.1(+). The 3.4 kb human *PKLR* minigene construct contained the proximal promoter and entire coding region of both R-PK and L-PK. A 3.1-kb human μLCR[19] (*kindly provided by G. Stamatoyannopoulos, University of Washington, Seattle, USA*) was replaced with an *Nru*I-*Hind*III fragment of pcDNA3.1(+), in which the cytomegalovirus promoter resided. The purified 6.5-kb μLCR/*PKLR* constructs were injected into fertilized pronuclei.

We examined the copy number of the transgene by Southern blot analysis using a 1.7-kb *Kpn*I-*Sfi*I genomic DNA fragment of the human *PKLR* gene labeled with DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics) as a probe. Ten micrograms of genomic DNA digested with *Bam*HI were used for each sample.

The genetic rescue of *Pk-1^{slc}* mice, which were homozygous for the missense mutation of the murine *PKLR* gene (Gly338Asp; G338D), was performed as follows: a mutant homozygote (*Pk-1^{slc}*) was mated with a PK transgenic mouse. Heterozygotes with the human RPK transgene were back-crossed with *Pk-1^{slc}*, and *Pk-1^{slc}* with the PK transgene were biochemically and hematologically examined. The murine *PKLR* gene was genotyped by polymerase chain reaction (PCR) analysis of tail DNA, as described elsewhere.[14] The transgene was detected by PCR with primers PK-Tg-F (5'-AGACTGGTGACAC-TAGTGTCTG-3') and PK-Tg-R (5'-GGATCACTGT-GATAATATGGTGG-3'), corresponding to sequences of the 3'-end of μLCR and the 5'-end of the *PKLR* gene. Aliquots of 0.5 μg of genomic DNA were amplified by PCR in 20-μL mixtures of 0.2 nmol/L dNTP with 10 pmol each of the primers and *ExTaq* polymerase (Takara Biochemicals, Japan). The reaction mixtures were subjected to 30 cycles of amplification consisting of 94°C for 20 seconds, 60°C for 20 seconds, and 72° C for 20 seconds in a GeneAmp PCR system 2400 (Roche Diagnostics, Switzerland).

RBC enzymes and glycolytic intermediates were measured by protocols described previously.[12] In order to separate human R-PK activity derived from the transgene
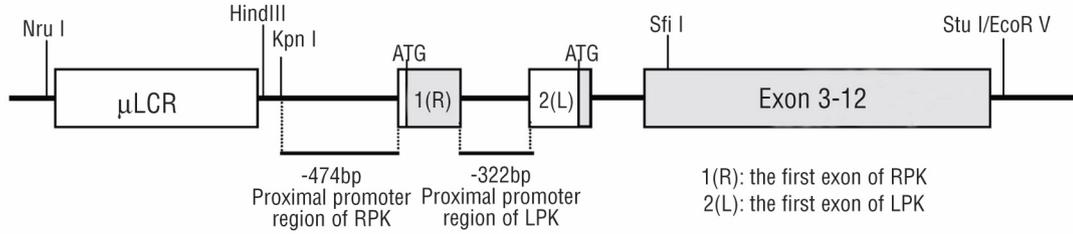
Figure 1. Structure of the human R-PK minigene with μLCR of the β-globin locus. A 1.7kb *KpnI-SfiI* genomic DNA fragment covering the 5'-flanking region of the human *PKLR* gene includes exons 1 and 2 of the human *PKLR* gene and the proximal promoters of both R-PK and L-PK. The 1.7kb R-PK cDNA fragment spanning from the *SfiI* site in exon 3 to the 3'-untranslated region was conjugated with the 1.7-kb *KpnI-SfiI* genomic DNA of the 5'-flanking region. Subsequently, a 3.1-kb human μLCR was subcloned at the 5'-end of the 3.4kb PK minigene, and the 6.5-kb μLCR/*PKLR* constructs were used for microinjection.

from endogenous murine R-PK, we utilized the zymogram of RBC lysate as follows: R-PK was partially purified by precipitation with 280 g/L ammonium sulfate, and applied on a thin-layer polyacrylamide gel. PK was visualized by activity staining as described elsewhere.[20] A TUNEL (terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling) assay was performed using an ApoTag *in situ* apoptosis detection kit (INTERGEN, Purchase, NY, USA), as described previously.[12] Apoptotic cells isolated from the spleen were analyzed using two-color flow cytometry,[11] using EPICS XL (Beckman-Coulter, Fullerton, CA, USA) and analyzed with EXPO32 ADC software (Beckman-Coulter). Annexin V-FITC and TER119 monoclonal antibody were obtained from PharMingen and Sigma, respectively.

Results

We obtained four founder mice, and three out of these four founders showed evident elevations of PK activity in RBC (Figure 2). There was no correlation between transgene copy numbers and PK activities, suggesting that

μLCR did not confer the position-independent, copy number-dependent expression of the PK transgenes. We back-crossed two founder mice (lanes 1 and 6 of Figure 1) with C57BL/6 mice and designated the transgenic lines as hRPK_lo and hRPK_hi; the RBC PK activity of these two lines was 87.8±4.8 and 121±1.9 IU/g Hb (mean±SD, n=3), respectively. The RBC PK activity of littermates carrying no transgene was 42.4±1.2 IU/g Hb, suggesting that the transgene expressed about equal or double the endogenous PK activity in RBC.

To confirm transgene expression in mice, we performed a zymogram of hemolysates prepared from RBC of transgenic mice with high levels of R-PK expression (hRPK_hi) (Figure 3). This system can separate human R-PK from endogenous PK activity as a slow-migrating band. As shown in the right lane, double bands corresponding to human and murine R-PK were visible, suggesting transgene expression in murine erythroid cells. Heterotetramers could not be observed in this system. Transgene expression was measured by the PK activities of RBC and tissue extracts from liver or muscle. PK activities of the two transgenic mice were elevated only in RBC and not in liver or muscle, indicating that the PK transgenes were
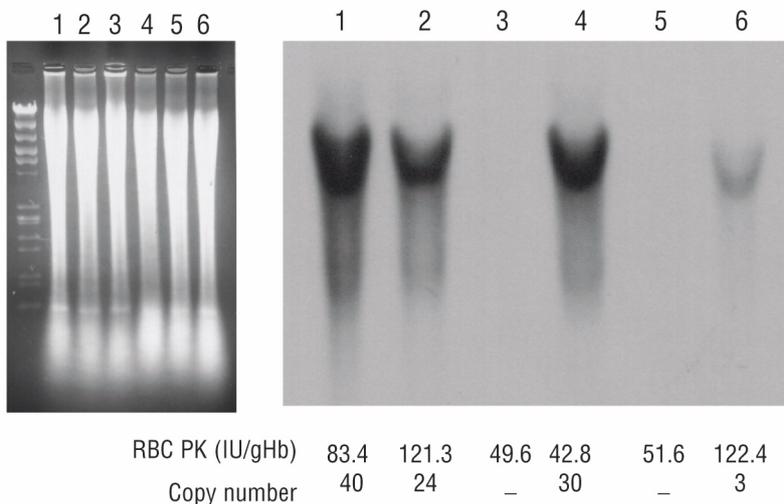

	1	2	3	4	5	6
RBC PK (IU/gHb)	83.4	121.3	49.6	42.8	51.6	122.4
Copy number	40	24	–	30	–	3

Figure 2. Copy numbers and RBC PK activities of the transgenic mice. Left panel, ethidium bromide staining of agarose gel running the *Bam*HI-digested genomic DNA; Right panel, the result of the Southern blot hybridization. Among six littermates, four mice had the transgene (lanes 1, 2, 4 and 6). Three of the founders showed about 2 to 3-fold elevations of PK activity in RBC. There is no correlation between transgene copy numbers and PK activities, suggesting that the μLCR does not confer position-independent, copy number-dependent expression of the PK transgenes.
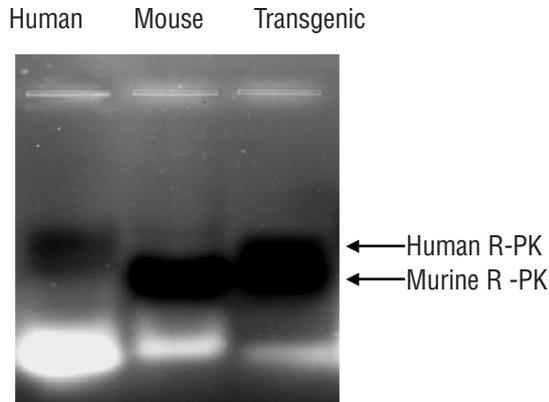
Human   Mouse   Transgenic

←Human R-PK
←Murine R-PK

Figure 3. Human PK activity demonstrated in RBC of PK transgenic mice To confirm transgene expression in mice, we performed a zymogram of hemolysates prepared from RBC. Partially purified R-PK is separated on a thin-layer polyacrylamide gel, and stained. This system can separate human R-PK from endogenous PK activity as a slow-migrating band. As shown in the right lane, double bands corresponding to human and murine R-PK are visible, suggesting transgene expression in murine RBC.

expressed in a tissue-specific manner (*data not shown*).

The therapeutic effects of the transgene are listed in Figure 4. R-PK activity, hemoglobin levels, reticulocyte counts, as well as spleen weights are compared between controls (CBA), mutant homozygotes (*Pk-1slc*), and the mice rescued with either low (hRPK_lo) or high (hRPK_hi) expression of the R-PK transgene. With a low expression of the transgene, R-PK activity reached about the same level as that in littermates (40.9±9.0 IU/g Hb, mean±SE, n=8). In the rescued mice with hRPK_lo, hemoglobin levels were 13.0±0.72 g/dL (mean±SE, n=8) with overt reticulocytosis (17±4.3%, mean±SE, n=8). In hRPK_hi mice, R-PK activity (76.0±10.8 IU/g Hb, mean±SE, n=8) reached twice that of control littermates. In this case, hemoglobin levels were about 15.1±0.31 g/dL (mean±SE, n=8) and reticulocyte counts were almost normal (3.1±0.26%, mean±SE, n=8). Even with a high expression level of the transgene, splenomegaly was still apparent.

We also looked at the glycolytic intermediates, pyruvate and ATP of the rescued mice as indicated in Figure 5a, b and c. 2,3-diphosphoglycerate (2,3-DPG), phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate accumulated up to levels 10-fold higher than normal in the mutant homozygote (Figure 5a,b). In contrast, the rescued mice showed apparent reduction of accumulated metabolites in an expression-level dependent manner, suggesting that overexpression of the normal PK gene ameliorated metabolic disturbances observed in mutants. It should be noted that the 2,3-DPG level of hRPK_hi mice decreased significantly to about 60% of the control level, probably due to overexpression of R-PK. Pyruvate,
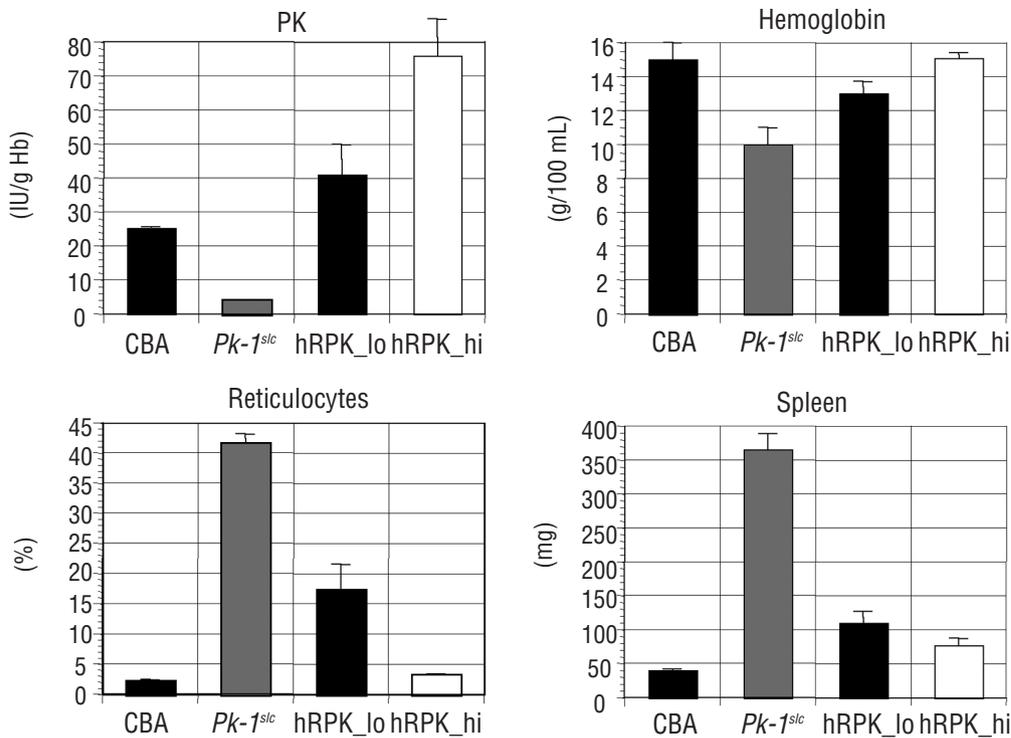

Figure 4. Expression levels of the transgene correlate with the therapeutic efficacy in R-PK deficient hemolytic anemia. R-PK activity, hemoglobin levels, reticulocyte counts, as well as spleen weights were compared between control (CBA), mutant homozygotes (*Pk-1slc*), and the mice rescued with low (hRPK_lo) and high (hRPK_hi) expression of R-PK transgene.
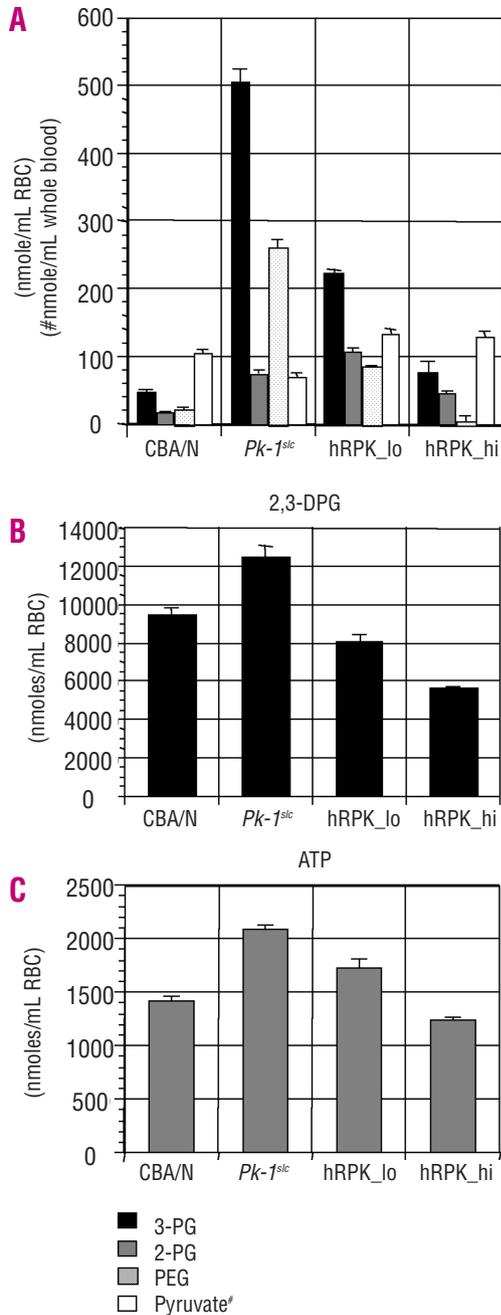
Figure 5. Changes of RBC glycolytic intermediates and adenosine triphosphate by transgene expression. The glycolytic intermediates and ATP level in RBC are compared between control (CBA), mutant homozygotes (*Pk-1^slc*), and the mice rescued with low (hRPK_lo) and high (hRPK_hi) R-PK transgenes. **A.** Phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate are accumulated up to values 10-fold higher than normal in the mutant homozygote, and significantly decreased in an expression-level dependent manner in the rescued mice. Pyruvate, the end product of the PK reaction is decreased in the mutant, and elevated to higher than control values in the rescued mice. **B.** The intracellular concentration of 2,3-DPG, a crucial regulator of oxygen affinity of hemoglobin, is increased in the mutant, and decreased to values less than control in the rescued mice. Its level in RBC of hRPK_hi was approximately 60% of the control value. **C.** Because of elevated reticulocyte counts, the mutant mice showed higher ATP values than the control value, as previously reported.[13] The transgene expression decreased ATP levels to subnormal values in an expression-level dependent manner.

the last product of aerobic glycolysis in RBC, was increased by the transgene up to about 120% of that of control mice. Because of elevated reticulocyte counts, the mutant mice showed higher ATP values than the control value, as previously reported.[13] Transgene expression normalized ATP level to the control value in an expression-level dependent manner (Figure 5c).

When we looked at apoptotic cells in the spleen by the TUNEL method, we observed enhanced apoptosis in the spleen of PK mutant mice, as previously reported.[12] These apoptotic cells accumulated in red pulp but not in white pulp, where B220-positive B cells were detected. In the spleen of rescued mice, TUNEL-positive cells were decreased, suggesting that these apoptotic cells were the PK-deficient erythroid progenitors (Figure 6).

Flow cytometric analysis using mononuclear cells prepared from the spleen showed that there were significantly fewer annexin V/Ter119-double positive cells in rescued mice than in mutant mice, confirming that cells of the erythroid lineage had escaped from apoptotic cell death by transgene expression. This provides direct evidence that R-PK deficiency causes apoptosis in erythroid cells and that both hemolysis and ineffective erythropoiesis account for anemia in PK deficiency (*data not shown*).

Discussion

There are 15 RBC enzyme deficiencies, which account for hereditary non-spherocytic hemolytic anemia in the glycolytic pathway, hexose monophosphate shunt, and nucleotide or glutathione metabolism.[2] Prenatal deaths have been reported in subjects severely deficient in glucosephosphate isomerase,[21,22] PK,[23-25] and hexokinase.[26] With the use of molecular biology techniques, prenatal genetic testing for RBC enzyme deficiencies has become possible, facilitating the management of severe prenatal hemolytic anemia.[27]

In spite of the remarkable progress made in diagnostic techniques, curative therapy for severe hemolytic anemia due to RBC enzyme disorders still remains undeveloped. HSCT has been used in animal models,[13,16,17] and a case of successful bone marrow transplantation has been reported recently.[15] Non-myeloablative HSCT seems a favorable strategy for the treatment of PK deficiency, as indicated in animal model studies, since erythroid progenitors with normal R-PK show a selective growth advantage.[13,17]

Enzyme-replacement therapy is another candidate for causative-targeted therapy of severe hemolytic anemia due to RBC enzyme defects. Ationu *et al.* reported a possible enzyme-replacement therapy for triose phosphate isomerase deficiency, a glycolytic enzyme defect which causes progressive neuromuscular impairment as well as hemolytic anemia.[28] Accumulation of dihydroxyacetone phosphate, a harmful glycolytic intermediate, might be partly responsible for the symptoms of triose phosphate
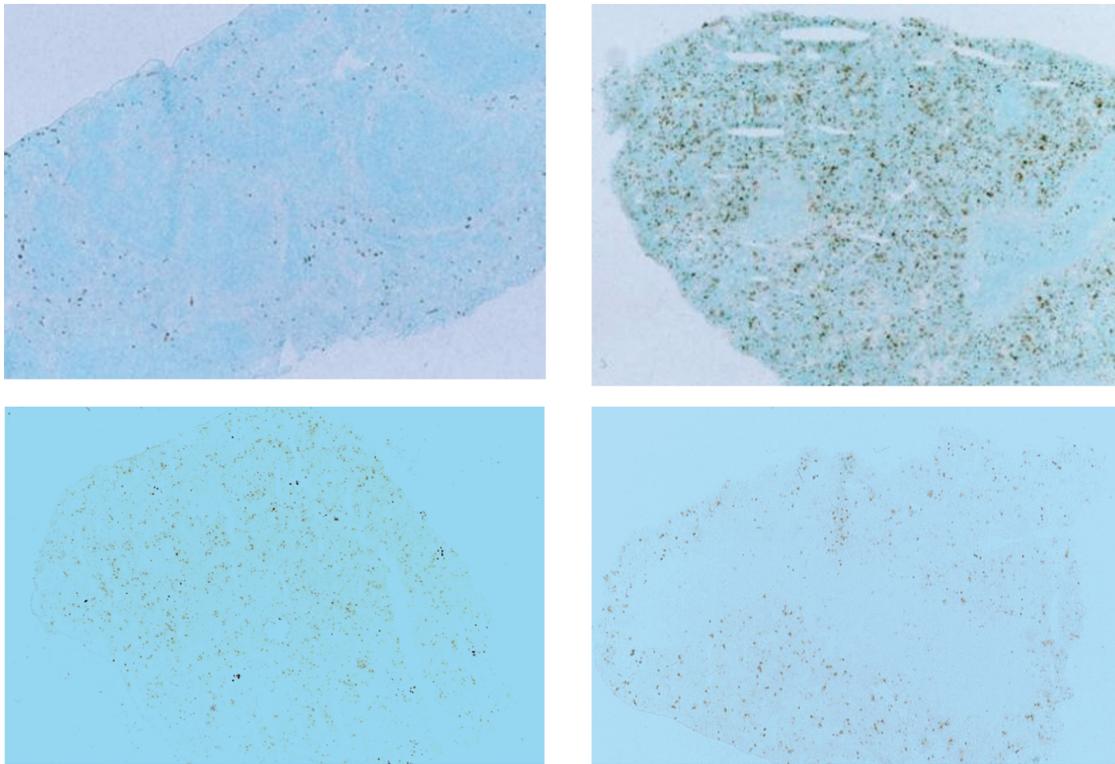
Figure 6. Spleen of the rescued mice; TUNEL staining. TUNEL-positive cells were significantly increased in the splenic red pulp of *Pk-1ˢˡᶜ* mice (upper right panel). There were a small number of positive cells in rescued mice (hRPK_lo, lower left; hRPK_hi, lower right).

CBA	*Pk-1ˢˡᶜ*
hRPK_lo	hRPK_hi

isomerase deficiency, and the phenotype is expectedly recovered by a slight increase of intracellular enzyme activity, as occurs in enzyme replacement for adenosine deaminase deficiency. However, it seems quite difficult to achieve a sustained therapeutic effect for PK deficiency by enzyme-replacement therapy, since the target level of enzyme activity for obvious clinical improvements is expected to be much higher than those of triose phosphate isomerase and adenosine deaminase deficiency.

Gene therapy has several theoretical advantages compared to HSCT, since severe complications such as rejection, infection, or graft-versus-host reaction can be avoided. In this study, we evaluated the therapeutic effectiveness of gene addition via a transgenic rescue strategy. We chose to rescue the R-PK deficient mice with the human PK-R gene for the following reasons: (i) we could separate human R-PK from murine R-PK by zymography; (ii) a tag, short amino acid sequence in the N- or C-terminal may affect enzymatic activity of R-PK derived from the transgene; (iii) we expected that a therapeutic effect would be achieved not by the heterotetramer between murine and human R-PK subunits but by the homotetramer of human R-PK subunits.

We evaluated the therapeutic effects of the transgene expression by hematologic and biochemical means, confirming that the hemolytic anemia of mutant mice was fully recovered with the high expression of the transgenic line, which showed about twice the endogenous R-PK activity of wild-type mice. However, the transgene with almost similar enzymatic activity as endogenous R-PK activity could not improve hemolysis with the homozygous mutant genes. It should be noted that the spleen of rescued mice, both hRPK_lo and hRPK_hi, showed substantial numbers of TUNEL-positive apoptotic erythroid cells. We postulate two possible explanations for the observation. Firstly, the forced expression of the transgene by μLCR is insufficient to overcome the variegated expression of exogenous R-PK in each erythroid cell; secondly, the μLCR cannot adequately activate the transgene in early erythroid progenitors. In order to activate the R-PK gene at the appropriate stage of erythroid differentiation, a more physiological enhancer/promoter system should be utilized. In this respect, it is necessary to elucidate the erythroid-specific enhancer of the human *PKLR* gene, which has been already identified in rats.[29]

It is most likely that the incorporation of mutant R-PK subunits into the tetramer may interfere with the full restoration of PK activity in some erythroid cells. Since over 80% of reported R-PK mutations are missense mutations,[2,3] it seems that a gene-replacement strategy which inactivates the endogenous mutant R-PK gene might be required to achieve the complete cure of PK deficiency.

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