PKR regulates proliferation, differentiation and survival of murine hematopoietic stem/progenitor cells

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Running title: PKR regulates hematopoietic stem/progenitor cells

Scientific category: Hematopoiesis and Stem Cells
Key Points:

- PKR may be an unrecognized but important regulator of HSPC cell fate
- PKR expression regulates the frequency of HSPCs in the bone marrow and their response to stress

Abstract:

PKR is an interferon-inducible, double-stranded RNA-activated kinase that initiates apoptosis in response to cellular stress. To determine the role of PKR in hematopoiesis, we developed transgenic mouse models that express either human PKR (TgPKR), or a dominant-negative PKR (TgDNPKR) mutant specifically in hematopoietic tissues. Significantly, peripheral blood counts from TgPKR mice decline with age in association with dysplastic marrow changes. Compared to WT, TgPKR mice have reduced colony forming capacity and colonies are more sensitive to hematopoietic stresses. In addition, TgPKR mice have fewer hematopoietic stem/progenitor cells (HSPC) and the percentage of quiescent (G0) HSPCs is increased. Importantly, treatment of TgPKR BM with a PKR inhibitor specifically rescues sensitivity to growth factor deprivation. In contrast, marrow from PKR knockout mice has increased colony formation potential and HSPCs are more actively proliferating and resistant to stress. Significantly, TgPKR HSPCs have increased expression of p21 and IRF-1 while cells from PKR knockout mice display mechanisms indicative of proliferation such as reduced eIF2α phosphorylation, increased Erk1/2 phosphorylation and increased CDK2 expression. Collectively, our data reveal that PKR may be an unrecognized but
important regulator of HSPC cell fate and may be play a role in the pathogenesis of bone marrow failure.
Introduction:

Multiple interactions between cytokines and growth factors with hematopoietic stem/progenitor cells (HSPCs) determine whether and how these cells remain viable to undergo self-renewal or commit to differentiation into specific lineages of mature blood cells in response to stress.1-3 4,5

The interferon-inducible, dsRNA-activated protein kinase, PKR, is a sentinel stress kinase that initiates the response to diverse cellular challenges such as viral infection, hematopoietic growth factor deprivation, inflammatory cytokines, Toll-like receptor ligands, and chemo-radiation therapy.6-8 We and others have reported that activated PKR can regulate proliferation and apoptosis by phosphorylation of eIF2α to inhibit new protein synthesis, activation of a PP2A-dependent Bcl2 dephosphorylation mechanism resulting in mitochondrial dysfunction and activation of signaling pathways such as NF-κB, p53 and STAT1.6,9-12 Significantly, loss of PKR expression/activity has been associated with increased growth of human breast carcinoma, non-small cell lung cancer (NSCLC), B-cell CLL, and T-cell ALL, suggesting that loss of PKR activity may contribute to increased growth and malignancy.13-16 In contrast, increased PKR activity may inhibit cell growth and enhance stress responses leading to apoptosis. In support of this notion, activated PKR has been reported to be increased in myeloid progenitor (CD34⁺CD33⁺) cells from myelodysplastic syndrome (MDS) patients and inhibition of PKR expression or activity can partially reverse the suppressive effects of IFNγ and TNFα cytokines on hematopoietic colony formation by normal or MDS-derived CD34⁺ cells.17,18 Taken together these results suggest that PKR may have a negative
regulatory role in hematopoiesis and potentially play a role in bone marrow failure states.

To test the role of PKR in the regulation of HSPC self-renewal, differentiation and in response to stress, we constructed novel transgenic mice that express either human PKR (TgPKR) or a catalytically null/dominant-negative PKR mutant (TgDNPKR) specifically in hematopoietic cells to compare hematopoiesis in these mice with WT or PKR null mice. TgPKR mice demonstrate a much reduced frequency of HSPCs that display decreased proliferation, reduced hematopoietic colony formation and increased sensitivity to apoptosis-inducing cell stress. Interestingly, we have discovered that PKR knockout (PKRKO) mice have increased numbers of HSPCs. In addition, PKRKO cells have increased CFU activity and are more resistant to cell death. These data indicate that PKR is an unrecognized but necessary negative regulator of HSPC fate and may play a role in bone marrow failure states.
**Materials and Methods:**

**Generation of human PKR and DNPKR transgenic mice:**

The cDNAs encoding either the human PKR or the human dominant-negative PKR(K296R) mutant were amplified and ligated into pcr2.1 using PCR primers that inserted a 5’ SfiI site and a 3’ NotI site (Invitrogen). The PKR or DNPKR ORF was ligated as a SfiI to NotI fragment into the HS21/45-vav expression vector (provided by Jerry M. Adams, WEHI, Melbourne, Australia) to generate vav-PKR or vav-DNPKR expression vectors. The University of Florida DNA sequencing core confirmed vector sequences, and expression of human PKR or DNPKR from the vav vector was tested for expression in FDC-P1 cells. Following HindIII digestion to remove the plasmid backbone, vav-PKR or vav-DNPKR was microinjected into pronuclei of C57BL/6 eggs by the University of Florida transgenic mouse core facility. Founder transgenic mice were identified by PCR with forward primer: 5’-TGTGCATCGGGGGTGCATGG-3’ and reverse primer: 5’-TCACGCTCCGCTTCTCGTT-3’ that generates a 510bp product. Founder mice were crossed with C57BL/6 mice to generate transgenic lines. Transgene negative siblings were used as wild type controls throughout the study. In addition, PKR knockout mice (PKRKO) were provided by Dr. Robert Silverman (Lerner Research Institute, Cleveland Clinic, Cleveland, OH). The study was approved by UF IACUC protocol #201102224.

**Immunoblotting and Real-time PCR:**

Snap-frozen mouse tissues were lysed and blotted with anti-human PKR (H00005610-M02, Abnova) or anti-mouse PKR (sc-6282, Santa Cruz). For protein
analysis of hematopoietic progenitor cells, BM cells pooled from 5 mice were lineage depleted and selected for c-Kit using MACS (Miltenyi Biotec); collected cells were lysed and immunoblotting was performed using either anti-ERK (#4695), phospho-ERK (#4370), eIF2α (#9722), phospho-eIF2α (#9721), Mcl1 (#5453) antibodies from cell signaling technology or IRF1 (sc-640), p21(sc-397), CDK2 (sc-163) antibodies from Santa Cruz biotech.

Total RNAs were isolated from using RNeasy reagent (Qiagen), and cDNA prepared (Applied Biosystems). TaqMan assays were done for human PKR (assay Hs00169345_m1) and mouse PKR (assay Mm01235644_m1) with β-actin as an endogenous control. PCR was carried out in a 7500 Fast RT-PCR system at 95°C for 20 seconds, 59°C for 20 seconds, 72°C for 20 seconds, for 40 cycles (Applied Biosystems).

**In vitro culture and survival assays of bone marrow progenitor cells:**

BM was harvested from femurs/tibias by flushing the marrow with 5ml PBS containing 10% FBS using a 27-gauge needle syringe. Red cells were lysed by ACK lysis buffer (0.15M NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA, PH 7.4) and nucleated cells resuspended in PBS containing 10% FBS and counted.

To measure hematopoietic colony formation, 2x10⁴ whole BM nucleated cells from wild-type, TgPKR, TgDNPKR, or PKRKO mice were plated in methylcellulose media (M3234; StemCell Technologies) containing 1x growth factor cocktail (3 U/ml EPO, 10 ng/ml mouse IL-3, 10 ng/ml mouse IL-6, and 50 ng/ml mouse SCF (R&D system). Colonies were enumerated following 7days using light microscopy.
To assess progenitor cell survival during growth factor starvation, BM cells were cultured in various dilutions of the growth factor cocktail (0.0675x to 1x) and CFUs measured. Apoptosis was determined by flow cytometry following annexin V staining. Where indicated, hematopoietic CFU was determined following culture of BM cells in either 300nM PKR inhibitor (Calbiochem) or various concentrations of TNFα or IFNγ. Radiation sensitivity was determined for CFUs following γ-irradiation of whole BM cells (1, 2, 3, or 4 Gy from a 60Co source delivered at 100 cGy/min).

**Colony forming unit-spleen (CFU-S) assays:**

Recipient mice 12 to 16 weeks of age were treated with 10 Gy of γ-irradiation. 2×10^4 whole, nucleated donor BM cells were suspended in 200 μL of 1xPBS with 10% bovine calf serum (Bovogen) and administered intravenously to recipient mice. Ten days later, spleens of recipient mice were harvested, fixed (60% ethanol, 30% chloroform, 10% acetic acid) and colonies counted.

**Flow cytometry and quiescence/cell cycle analysis:**

Bone marrow mononuclear cells were stained with a fluorochrome-conjugated lineage antibody cocktail (CD3, B220, Ter119, CD11b, and Gr-1) (BD Pharmingen), Sca-1, c-Kit, CD16/32, CD34, CD45, CD11b, and Gr-1 (ebioscience) and analyzed using a LSR II flow cytometer (BD Biosciences). To measure quiescence, Lin-c-Kit+ cells were isolated using MACS columns (Miltenyi Biotec), fixed in 1% paraformaldehyde, permeabilized with 0.1% Triton X-100, stained with PI (Invitrogen) and anti-Ki-67 PE (BD Pharmingen), and analyzed by flow cytometry. To measure proliferation and differentiation of stem/progenitor cells, 5×10^4 Lin-c-Kit+ cells isolated were cultured in RPMI1640 media containing 1x growth factors. After 0, 2, 4, 6, and 8 days, the cultured
cells were stained with fluorochrome-conjugated antibodies to c-Kit, CD11b and Gr-1 and cell numbers determined by flow cytometry.

**Complete blood count and pathology:**

Peripheral blood samples were collected from cheek bleed and complete blood counts obtained using a Hemavet analyzer (Drew Scientific, Inc.).

Histopathologic examination was performed on H&E- stained specimens prepared from thymus, lymph nodes, spleen, kidney, lung, heart, liver fixed in alcoholic-paraformaldehyde solution and decalcified femur specimens were fixed in neutral-buffered 10% formalin. Bouin-fixed, paraffin-embedded tissues were sectioned and deparaffinized. Sections were counterstained with H&E. Bone marrow smears were prepared from femurs and stained with Wright-Geimsa.

Slides were viewed with an Olympus upright microscope equipped with U plan Fluorite objectives at 40x/0.75 and 100xoil/1.30 (Olympus, Tokyo, Japan) and Cytoseal mounting medium. Images were acquired using a DP71 digital camera with DP-BSW-V3.1 camera control software and processed with Adobe Photoshop version 7.0.

**Statistical analysis:**

All data are presented as the mean ± SEM. Statistical significance ($P < 0.05$) was determined by t test using Graphpad Prism v. 5.0.
Results:

Hematopoietic-tissue specific expression of human PKR in mouse.

Transgenic mice were generated that express full-length human PKR (TgPKR) or a catalytically null, dominant-negative PKR(K296R) mutant (TgDNPKR) driven by the vav transcriptional elements to direct gene expression specifically in hematopoietic tissues (Figure 1A).\textsuperscript{19,20} Founder TgPKR or TgDNPKR mice were identified by PCR of genomic DNA (Figure 1B). Importantly, human PKR or DNPKR is highly expressed in thymus, spleen and bone marrow (BM) compared to non-hematopoietic tissues such as small intestine, liver, or kidney tissues as demonstrated by real-time quantitative PCR and Western blotting (Figure 1C and 1D).

Transgenic expression of PKR induces histological features of bone marrow dysplasia.

Bone marrow from TgPKR or TgDNPKR mice was examined for histopathological abnormalities. Strikingly, more than 80\% (16 out 19) of TgPKR mice display mild to moderate dysplastic changes in either erythroid, myeloid, or megakaryocytic cells (Figure 2A to 2D, green arrowheads). Furthermore, as TgPKR mice age, megakaryocytic dysplasia increases from 40\% in 3-month-old to 65\% in the 10-month-old group (200 megakaryocytes counted per mouse; Figure 2E). In addition, TgPKR mice demonstrate increased marrow cellularity with an increase in myeloid and lymphoid blasts and mild necrosis. No significant BM abnormalities have yet been identified in TgDNPKR mice, indicating that the effect of PKR on hematopoiesis may
require kinase activity (data not shown). In addition, a significant number of TgPKR mice develop dermatitis that may indicate a condition of chronic inflammation (data not shown). For comparison, we examined PKR knockout mice (PKRKO). Interestingly, while no significant differences in BM pathology were apparent, PKRKO mice display increased body weight of littermates (average weight: 39.2g vs. 32.7g, n = 10, P < 0.01), and a larger average spleen size than WT mice when measured at 5 to 6 months of age (PKRKO (n = 7): 0.98g vs. WT (n =11): 0.86g; P = 0.034).

Analysis of peripheral blood indicates that TgPKR mice have significantly lower total white blood cell, neutrophil, lymphocyte, platelet and hemoglobin counts compared to WT mice, indicating defective hematopoiesis (Table 1). Furthermore, this trend becomes exaggerated as mice age for 16-18 months. In contrast, WBC, platelet and hemoglobin levels in PKRKO or TgDNPKR increase compared to WT mice and differences between the CBC of TgPKR and WT or TgDNPKR mice becomes more pronounced as mice age (Table 1). Taken together, these findings indicate that aberrant activation of PKR in the hematopoietic compartment may play a role in bone marrow failure states. In addition, the absence of expression/activity of PKR during aging is clearly associated with increased numbers of hematopoietic cells.

**PKR expression affects proliferation and differentiation of hematopoietic stem/progenitor cells.**

To investigate whether aberrant expression/activation of PKR may affect proliferation and/or differentiation of HSPCs and to explain the changes observed in peripheral or BM, we carried out hematopoietic colony forming cell (CFC) assays using
BM isolated from TgPKR, TgDNPKR, PKRKO and WT mice. Results show HSPCs of TgPKR mice give rise to significantly fewer CFUs that are smaller in size compared to WT control mice (Figure 3A). Furthermore, PKRKO BM contains increased numbers of CFUs compared to WT mice, a previously unrecognized phenotype for the PKR null mice (Figure 3A). These findings indicate that PKR expression may directly affect growth of HSPCs.

Breakdown of hematopoietic colony types show that the TgPKR mice have impaired formation of multipotent colony-forming units (CFU-GEMM), bipotent granulocyte macrophage (CFU-GM), unipotent granulocyte (CFU-G), macrophage (CFU-M) and burst-forming unit erythroid (BFU-E) colonies, indicating a global impairment of progenitor cell differentiation toward the myeloid lineage (Figure 3B to 3F). By contrast, BM from PKRKO mice display an increase in the frequency of all myeloid lineages including CFU-GEMM, CFU-GM, CFU-G, CFU-M, CFU-E and BFU-E (Figure 3B to F).

To assess the function of HSPCs we performed an in vivo colony-forming spleen (CFU-S) assay. Consistent with the findings of the in vitro CFC assay, TgPKR BM gave rise to significantly fewer while PKRKO BM gave rise to significantly more CFU-S compared to WT (Fig 3G and H). These results indicate that the HSPCs from TgPKR mice are less frequent and/or have an impaired capacity to reconstitute multi-lineage hematopoiesis compared to cells from PKRKO mice. Thus, PKR may have a regulatory role in the full and robust proliferation and differentiation of HSPCs.
**Frequencies of hematopoietic stem/progenitor cell populations are altered in BM of TgPKR and PKRKO mice.**

To investigate whether PKR affects HSPCs populations *in vivo*, BM cells were isolated and analyzed by flow cytometry (Figure 4A). TgPKR mice were found to have a significant decrease in the number of hematopoietic stem cells (LSK\(^{-}\),Sca1\(^{+}\),cKit\(^{+}\)) as well as common myeloid progenitor cells (CMP: Lin\(^{-}\),Sca1\(^{-}\),cKit\(^{+}\),CD34\(^{+}\),CD16/32\(^{low}\)) when compared to WT mice (Figure 4B and 4C). Furthermore, both megakaryocyte-erythroid progenitor (MEP: Lin\(^{-}\),Sca1\(^{-}\),cKit\(^{+}\) CD16/32\(^{low}\),CD34\(^{-}\)) and granulocyte-macrophage progenitor (GMP: Lin\(^{-}\),Sca1\(^{-}\),cKit\(^{+}\) CD16/32\(^{+}\),CD34\(^{+}\)) populations are decreased in TgPKR mice compared to WT mice. In contrast, both TgDNPKR and PKRKO mice have significantly elevated numbers of BM derived HSPCs (Figures 4A to 4E). While the number of HSPCs varies greatly between WT, TgPKR, TgDNPKR and PKRKO mice, the distribution of CMP, GMP and MEP populations remains similar (Figure 4A). Therefore, the inhibitory effect of PKR expression/activation on the differentiation of BM HSPCs does not affect a specific lineage.

To assess whether the development of mature myeloid cells is affected by PKR expression, BM cells from WT, TgPKR, TgDNPKR or PKRKO mice were stained for mature myeloid lineage markers to measure the CD45\(^{+}\)CD11b\(^{-}\) and CD45\(^{+}\)Gr-1\(^{+}\) populations. Significantly, BM from TgPKR mice display a 17% decrease in the CD45\(^{+}\)CD11b\(^{-}\) and an 18% decrease in the CD45\(^{+}\)Gr-1\(^{+}\) population compared to WT control (Figure 4F and 4G). In contrast, BM from PKRKO mice display a 17% and 25% increase, respectively, in CD45\(^{+}\)CD11b\(^{-}\) and CD45\(^{+}\)Gr-1\(^{+}\) populations compared to WT
mice (Figure 4F and G). Collectively, these results confirm that PKR is a necessary regulator of HSPCs.

**PKR regulates cell cycle distribution and quiescence status of hematopoietic stem/progenitor cells.**

Since impaired function of HSPCs may result from alterations in proliferation, we performed cell cycle analysis by measuring Ki67 to distinguish dividing from resting/quiescent (G0) cells. Significantly, the HSPC Lin−cKit+ population of 3-5 month old TgPKR mice has a higher percentage of quiescent G0 cells with fewer proliferating cells in S+G2/M phases as compared to WT mice (Figure 5A). In addition, results indicate that PKR activity is required for this effect, since the cell cycle distribution of TgDNPKR and WT is similar. However, Lin-cKit+ cells from PKRKO mice have a markedly reduced frequency of quiescent (G0) and a higher percent of proliferating (S + G2/M) cells compared to WT (Figure 5A).

Consistent with an inhibitory effect on the cell cycle, increased PKR expression inhibits HSPC proliferation when cultured in vitro. For example, after 8 days growth there is a 20% decrease in the number TgPKR Lin−cKit+ cells compared to WT cells (Figure 5B, $P < 0.01$). Conversely, HSPCs isolated from PKRKO mice have a significantly higher proliferation rate with 42% more cells than WT after 8 days in culture (Figure 5B, $P < 0.01$). This inhibitory effect of PKR on HSPC proliferation translates to terminally differentiated cells where TgPKR Lin−cKit+ cells give rise to 25% fewer, while the PKRKO cells produce 47% more CD11b+Gr+ cells (Figure 5C).
To investigate the mechanism(s) involved in PKR-mediated inhibition of HSPC proliferation, western blotting was performed on Lin-cKit+ cell lysates. Significantly, HSPCs from TgPKR mice display elevated levels of several potent negative regulators of proliferation including phospho-eIF2α, IRF-1 and p21. In contrast, cells from TgDNPKR or PKRKO mice display decreased IRF-1 and increased phosphorylated Erk1/2 (Figure 5D). Furthermore, HSPCs from PKRKO mice have decreased CDK2 (Figure 5D). These findings support previous reports that PKR is required for eIF2α and IRF-1 induced cell growth inhibition\textsuperscript{6,21,22}

**PKR regulates stress survival of HSPCs in response to growth factor deprivation, inflammatory cytokines or irradiation.**

To test whether PKR may play a role in regulating the response to stress in HSPCs, we measured cell survival following application of various stresses in clonogenic assays. First, we investigated whether PKR expression may affect the sensitivity to hematopoietic growth factor (HGF) deprivation. Following culture of HSPCs for increasing time in the absence of factors, cells were plated in methylcellulose containing SCF, IL-3, IL-6 and EPO and colonies scored after 7 days. HSPCs isolated from TgPKR mice are significantly more sensitive to HGF deprivation with decreased survival exhibiting a half-life (i.e. time of deprivation at which 50% progenitor cell colonies survive) of 28 hours compared to 36 hours for WT cells ($P < 0.01$, Figure 6A). Importantly, cells from the PKRKO mice were highly resistant to factor deprivation, exhibiting a half-life of 54 hours ($P < 0.01$; Figure 6A). In addition, Annexin V analysis confirms that reduced survival results from increased apoptosis in TgPKR
and reduced apoptosis in TgDNPKR and PKRKO cells (Figure 6B). Interestingly, PKRKO cells display higher CFU efficiency in lower concentrations of HGFs (left shift of curve) compared to WT cells. In contrast, cells from TgPKR mice require more than four times the dose of HGFs to achieve the same 100% CFU efficiency as WT cells (i.e. right shift of curve; Figure 4C).

As a control, we tested whether a pharmacological PKR inhibitor (PK Ri) could rescue reduced CFU activity of TgPKR cells. Our results demonstrate that 300nM PK Ri can significantly enhance colony formation of TgPKR cells in response to low concentrations of HGFs (Figure 6D). Although colony formation was moderately elevated by PK Ri treatment of WT cells, little or no effect was observed in TgDNPKR or PKRKO BM (Figure 6E to G). These data strongly indicate that PKR activity is required for its inhibitory effect on hematopoiesis.

To examine the role of PKR during the response to inflammatory cytokines, BM cells were plated in methylcellulose medium containing HGFs with increasing concentrations of IFNγ or TNFα. Both cytokines suppress CFU more potently in cells from TgPKR compared to WT mice (Figures 6H and I). Furthermore, the inhibitory effect of these cytokines is significantly reduced on TgDNPKR or PKRKO HSPCs, suggesting that activation of PKR is necessary for the myelosuppressive effect of inflammatory cytokines.

We next assessed the effect of PKR on radiosensitivity of BM cells in vitro. Cells were plated as above and immediately exposed to increasing doses of gamma irradiation. HSPCs from TgPKR mice were found to be significantly more sensitive to irradiation as evidenced by a lower D37 (the radiation dose for a survival of 37%) of 1.48
Gy ($P < 0.05$) and a reduced SF$_{2\text{Gy}}$ (surviving fraction at 2Gy irradiation) of only 22.0% ($P < 0.05$) as compared with a $D_{37}$ of 2.05 Gy and SF$_{2\text{Gy}}$ of 41.6% for WT cells (Figure 6J). Moreover, HSPCs from the PKRKO mice are highly resistant to radiation exposure with a $D_{37}$ of 2.68 Gy ($P < 0.05$) and an SF$_{2\text{Gy}}$ of 60.7% ($P < 0.05$), respectively (Figure 6J). Thus, inhibition or loss of expression of PKR may prevent the myelosuppressive effect of irradiation.
Discussion

Loss of PKR expression/activity has been reported in hematological malignancies including B cell CLL and T cell ALL whereas activated PKR is reported to occur in close association with MDS in primary patient samples.\textsuperscript{13,15,18} This suggests that PKR may play a role in regulating normal and malignant hematopoiesis. Therefore, we developed mice expressing either a human PKR or DNPKR transgene specifically in hematopoietic tissues and examined hematopoiesis in these mice and in PKR knockout mice. While a CMV-driven PKR transgenic mouse model has been reported, no effect was noted on hematopoiesis since the transgene was not expressed in hematopoietic tissues.\textsuperscript{23} Significantly, results from our vav-driven transgenic PKR mouse clearly indicate that PKR plays a role in regulating hematopoiesis. Specifically, increased PKR expression/activation in TgPKR cells leads to features of defective hematopoiesis and bone marrow failure as demonstrated by mild pancytopenia of all peripheral blood elements. In addition, the BM of TgPKR mice contains dysplastic appearing cells, reduced HSPC populations, reduced hematopoietic colony forming capacity, and increased sensitivity to various stresses that mediate cell death when compared to cells from WT mice. Furthermore, an increased percentage of HSPCs isolated from TgPKR mice are quiescent (G\textsubscript{0}) compared to WT mice. Since an effective exit from quiescence into S+G2/M phases is necessary for the expansion of the HSPC pool, the lower proliferation rate of the TgPKR HSPCs may, at least in part, explain the reduced numbers and frequency of HSPC populations including LSK, CMP, GMP and MEP observed in these mice. Furthermore, suppression of myeloid differentiation with
increased sensitivity to stress mediated apoptosis may in turn cause inefficient terminal
differentiation of blood cells, resulting in lower peripheral blood cell counts as observed.

Significantly, PKR null mice display markedly increased numbers of HSPCs with
an increased potential for hematopoietic colony formation. To account for this, results
indicate that the frequency of HSPCs in active proliferation, as measured by Ki67
expression, is increased. We hypothesize that activated PKR inhibits hematopoiesis by
mechanism(s) involving reduced protein synthesis and decreased survival following
stress. In support of this notion, bone marrow progenitor cells isolated from PKRKO
mice express decreased basal levels of phospho-eIF2α, increased levels of phospho-
Erk1/2 and increased CDK2 that indicate mechanisms of proliferation are activated in
these cells. Thus, dynamic activation/inactivation of PKR during homeostasis is
expected to regulate HSPC numbers.

Interestingly, despite having increased numbers of HSPCs and displaying
increased CFU potential, PKRKO mice have no differences in BM histopathology
compared to WT mice. However, they do display significantly elevated peripheral blood
cell counts with aging compared with WT mice. To account for this, we suspect an
uncharacterized feedback mechanism may limit inappropriate differentiation of PKRKO
HSPCs under basal (non-stress) conditions. Importantly, the effect of PKR on
hematopoiesis becomes most evident under times of cell stress. For example, BM cells
from PKR knockout mice are significantly more resistant to cell death resulting from
growth factor starvation, inflammatory cytokine treatment and irradiation. Thus, we
propose that the primary role for PKR in HSPCs is to potentiate the stress response and
that loss of PKR activity may lead to increased cell survival following stress, while over-
expression and activation may decrease cell viability. In support of this, others have reported that PKR null mice are protected from DMBA-induced bone marrow toxicity leading to hypocellularity. Furthermore, although PKR null mice have not been reported to develop spontaneous tumors or leukemia during their normal 2 year lifespan, we hypothesize that these mice will be more susceptible to transformation by genotoxic stimuli or oncogenic driver mutations since the mice are predisposed to withstand stress.

Collectively, results here indicate that PKR may be a critical factor in the pathogenesis of some bone marrow failure states, potentially including MDS. Increased activity and aberrant subcellular location of PKR have been reported in CD34+ myeloid progenitor cells isolated from high-risk MDS patients, while loss of PKR expression/activity has been reported in BM cells from leukemia patients. In the present study, results indicate that the majority (> 80%) of TgPKR mice develop dysplastic BM changes as in the development of bone marrow failure syndromes. In addition, TgPKR mice display increased marrow cellularity with greater numbers of myeloid and lymphoid blasts as well as mild necrosis. This phenomenon may have clinical relevance because BM from MDS patients typically display hypercellularity (due to inhibition of terminal differentiation) with enhanced apoptosis/necrosis. These results suggest that inhibition of PKR may be a novel therapeutic approach for the treatment of marrow failure syndromes which display elevated levels of PKR. In addition, in bone marrow transplantation and therapy-related bone marrow suppression/ablation, inhibition of PKR activity might be a novel method to accelerate hematopoietic reconstitution.
Acknowledgements

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Authorship contributions

X.L. and R.L.B performed research, analyzed and interpreted data and wrote the manuscript. X.C., M. B. and M. K. R. performed research. W. S. M. analyzed and interpreted data and wrote the manuscript.

Conflict of interest disclosure

The authors have no conflicts of interest to declare.
References


Table 1. Peripheral blood analyses of WT, TgPKR, TgDNPKR and PKRKO mice

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<td>TgPKR</td>
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<td>TgPKR</td>
<td>5.57 ± 1.19</td>
<td>1.16 ± 0.37</td>
<td>4.03 ± 0.99</td>
<td>11.02 ± 0.53</td>
<td>421.6 ± 85.09</td>
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<td>P</td>
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<td>0.3197</td>
<td>0.0068</td>
<td>0.0028</td>
<td>0.0033</td>
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<td>TgDNPKR</td>
<td>15.32 ± 0.62</td>
<td>3.52 ± 0.31</td>
<td>11.48 ± 0.55</td>
<td>14.50 ± 0.16</td>
<td>922.8 ± 47.73</td>
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<td>0.0020</td>
<td>0.0004</td>
<td>0.0019</td>
<td>0.0387</td>
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<td>PKRKO</td>
<td>16.48 ± 1.83</td>
<td>3.544 ± 0.77</td>
<td>11.86 ± 1.01</td>
<td>12.72 ± 0.42</td>
<td>907.2 ± 43.90</td>
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<td>P</td>
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<td>0.0492</td>
<td>0.0048</td>
<td>0.1719</td>
<td>0.0485</td>
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<td><strong>16-18 months old mice:</strong></td>
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<td>WT</td>
<td>10.47±1.40</td>
<td>2.09±0.28</td>
<td>8.38±1.12</td>
<td>11.14±0.36</td>
<td>717±43.4</td>
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<td>(n=5)</td>
<td>(n=5)</td>
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<tr>
<td>TgPKR</td>
<td>5.18±1.22</td>
<td>0.89±0.24</td>
<td>4.29±1.00</td>
<td>7.9±0.91</td>
<td>401.2±54.9</td>
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<td>0.005</td>
<td>0.013</td>
<td>0.005</td>
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<tr>
<td>TgDNPKR</td>
<td>15.42±1.23</td>
<td>3.84±0.40</td>
<td>11.58±1.05</td>
<td>13.1±0.34</td>
<td>855.8±35.6</td>
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<td>0.003</td>
<td>0.035</td>
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<tr>
<td>PKRKO</td>
<td>17.34±1.86</td>
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<td>14.56±0.16</td>
<td>924±39.08</td>
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<tr>
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<td>0.009</td>
<td>0.002</td>
<td>0.024</td>
<td>&lt;0.001</td>
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Complete blood count analyses were performed with TgPKR, TgDNPKR mice and their WT or PKRKO mice. WBC indicates white blood cells; NE, neutrophil; LY, lymphocyte; HGB, hemoglobin; PLT, platelet. Statistical significance (P) compared to WT was calculated by t test.
Figure Legends

Figure 1. Mice are generated that express either a human PKR or dominant-negative PKR mutant transgene exclusively in hematopoietic tissues. (A) Schematic of construct used for pronuclear injection. (B) PCR analysis of tail DNA to detect transgenic mice using primers specific for human PKR. PCR for β-actin was used as an endogenous control. Lanes 4 and 5 are transgenic pups others are wild type siblings. (C) Real-time PCR assays of human PKR or DNPKR expression in the indicated tissues of transgenic mice. Data represent relative mRNA levels (mean ± SEM, n = 3) normalized to mouse β-actin in arbitrary units. (D) Total cell lysates were analyzed by western blot for human PKR or DNPKR protein expression in the tissues indicated.

Figure 2. Transgenic expression of PKR induces bone marrow dysplasia in TgPKR mice. (A and B) Hematoxylin and eosin staining of paraffin-embedded sections of femur (for bone marrow) from WT control and TgPKR mice as indicated. White arrows point to normal megakaryocytes. Green arrows indicate dysplastic megakaryocytes with single nuclear lobe or with multiple separated nuclear lobes. (C and D) Wright-Giemsa staining of bone marrow smears from WT control or TgPKR mice as indicated. White arrows indicate normal megakaryocytes. Green arrow indicates a dysplastic megakaryocyte with multiple separated nuclear lobes. (E) Graph showing percentage of dysplastic megakaryocytes in 3 vs.10 months old WT or TgPKR mice.
Five mice of each genotype were analyzed with 200 megakaryocytes counted per specimen.

**Figure 3. PKR regulates clonogenic potential of bone marrow progenitors both in vitro and in vivo.** (A) Representative colonies following culture of WT, TgPKR, TgDNPKR, or PKRKO BM cells from 3 - 5 months old mice in methylcellulose for 7 days. (B to F) Unfractionated BM cells (2x10^4 for B, C, D, and E, 10^5 for F) from WT, TgPKR, TgDNPKR, or PKRKO mice were plated and resulting CFU-GEMM, CFU-GM, CFU-M, CFU-G and BFU-E counted. BM from five mice of each genotype was assayed and mean ± SEM graphed. (G) Representative examples of macroscopic spleen colonies (CFU-S) from WT irradiated recipients injected with 2x10^4 unfractionated bone marrow cells either from WT, TgPKR, TgDNPKR, or PKRKO mice as indicated. (H) Colonies were counted 10 days after transplantation. Graphs represent mean ± SEM (n = 4 donor mice of each genotype).

**Figure 4. In vivo sizes of hematopoietic stem/progenitor cell compartments and development of granulocyte/monocytes is altered in TgPKR transgenic and PKR knockout mice.** (A) BM from 3-5 months old mice was stained for lineage markers Lin, Sca-1, c-Kit, CD34 and CD16/32. The CD34 and CD16/32 staining of the Lin<sup>-</sup>, c-Kit<sup>+</sup> subset was used to discriminate between hematopoietic progenitor cell populations. (B) Graph depicting the relative frequency of LSK hematopoietic stem cells in total BM. (C) The frequency of common myeloid progenitor (CMP) cells in BM. (D) The frequency of megakaryocyte-erythroid progenitor (MEP) cells in total BM. (E) The frequency of
granulocyte-macrophage progenitor (GMP) cells in total BM. (F) BM was stained for the monocyte marker CD11b and measured by flow cytometry. Graph shows the frequency of CD45⁺CD11b⁺ monocytes in total BM. (G) Graph of the average relative frequency of granulocytes (CD45⁺Gr-1⁺) in total BM cells. For all graphs the average data from 5 mice of each genotype is represented.

Figure 5. PKR regulates quiescence and cell cycle progression of HSPCs. (A) Flow cytometry analysis of DNA content and Ki67 expression in Lin⁻c-Kit⁺ bone marrow cells from WT, TgPKR, TgDNPKR or PKRKO mice. Numbers on the plot are the frequency of cells in the indicated cell cycle phases. (B) Proliferation of Lin⁻c-Kit⁺ bone marrow cells from WT, TgPKR, TgDNPKR or PKRKO mice in vitro under standard growth conditions was measured by flow cytometry (n=5). (C) Differentiation of Lin⁻c-Kit⁺ cells from WT, TgPKR, TgDNPKR or PKRKO mice into CD11b+Gr1+cells was measured by flow cytometry following culture under standard growth conditions. (D) Western blotting of Lin⁻c-Kit⁺ bone marrow cells from WT, TgPKR, TgDNPKR or PKRKO mice to investigate the mechanisms of PKR-dependent proliferation and cell cycle regulation. The cells from 5 mice for each genotype were pooled for western blotting.

Figure 6. PKR regulates survival of clonogenic bone marrow progenitor cells in vitro following hematopoietic growth factor deprivation, inflammatory cytokine treatment or γ-irradiation. The colony formation data are expressed as percent of maximal numbers of colonies. Mean actual maximal colony counts per 2x10⁴ cells were
WT=70.4, TgPKR=53.4, TgDNPKR=77, PKRKO=111.6. (A) BM cells were cultured in media without growth factors for the indicated times. Cells (2x10⁴) were then plated in methylcellulose-based media containing 1x growth factors (50 ng/ml SCF, 10ng/ml IL-3, 10ng/ml IL-6 and 3U/ml EPO) and colonies scored after 7-days (n = 3). (B) Lin⁻c-Kit⁺ cells were isolated from BM of WT, TgPKR, TgDNPKR or PKRKO mice and cultured in media without growth factors. At the indicated times, measurement of Annexin V staining was performed by flow cytometry to determine apoptosis. (n =3). (C) BM Cells (2x10⁴) were plated in methylcellulose-based media containing various concentrations of the hematopoietic growth factor cocktail (0.0675x to 1x, 1x = 50 ng/ml SCF, 10ng/ml IL-3, 10ng/ml IL-6 and 3U/ml EPO) and hematopoietic colony formation scored. (D – G) Cells (2x10⁴) were plated in methylcellulose-based media containing various concentrations of the hematopoietic growth factor cocktail and 300nM of either PKR inhibitor or inactive control. (H) Hematopoietic colony formation was assayed in medium containing 1x growth factors and the indicated concentration of IFNγ. (I) Hematopoietic colony formation was assayed in medium containing 1x growth factors and the indicated concentration of TNFα. (J) Hematopoietic colony formation was assayed following irradiation of BM cells with the indicated doses of γ-irradiation. (For all colony formation assays, colonies were scored after 7 days growth and results are the average of BM from 3 mice (n = 3) for each genotype.)
Figure 1

A

vav element  hPKR / hDNPKR cDNA  vav element

SV40 intron  SV40 PA

B

hPKR transgene

WB: hPKR

β-Actin

β-Actin

C

TgPKR/TgDNPKR

WT

BM  Spleen  Thymus  Intestine  Liver  Kidney  BM  Thymus

0  0.5  1  1.5  2  2.5  3  3.5  4

D

WT

Thymus  Spleen  K562  FDC-P1  Thymus  Liver  Kidney

WB: hPKR

Bone Marrow

TgPKR

Thymus  Spleen  K562  FDC-P1  Thymus  Liver  Kidney

WT  TgPKR

WB: hPKR

Bone Marrow

WT  TgDNPKR

Thymus  Spleen  K562  FDC-P1  Thymus  Liver  Kidney

WT  TgPKR

WB: hPKR

Bone Marrow

WT  TgDNPKR

Thymus  Spleen  K562  FDC-P1  Thymus  Liver  Kidney

WT  TgPKR

WB: hPKR

Bone Marrow

WT  TgDNPKR

Thymus  Spleen  K562  FDC-P1  Thymus  Liver  Kidney

WT  TgPKR

WB: hPKR
Figure 2

A. H&E staining of femur section
   - WT
   - TgPKR

B. Wright Giemsa staining of BM smear
   - WT
   - TgPKR
Figure 2

E

Frequency (%) of dysplastic megakaryocyte

WT

TgPKR

P<0.01

P<0.01

3 months

10 months
Figure 3

A

WT

TgPKR

TgDNPKR

PKRKO
Figure 3

G

WT  TgPKR  TgDNPKR  PKRKO

H

CFUs number/2x10^4 BM cells

P=0.01*
P=0.005*
Figure 4
A

WT:

TgPKR:

TgDNPKR:

PKRKO:
Figure 4

B. LSK (Lin\(^{-}\)Sca1\(^{+}\)cKit\(^{+}\))

- P = 0.018
- P = 0.045
- P = 0.035

C. CMP

- P = 0.027
- P = 0.038

D. MEP

- P = 0.035
- P = 0.026
- P = 0.003

E. GMP

- P = 0.018
- P = 0.033

F. CD11b\(^{+}\)CD45\(^{+}\)

- P = 0.011
- P = 0.042
- P = 0.029

G. Gr1\(^{+}\)CD45\(^{+}\)

- P = 0.014
- P = 0.044
Figure 5

(A) Flow cytometry analysis of cell cycle distribution and Ki67 expression in different genotypes:

**WT**
- **G1:** 57.5%
- **S+G2/M:** 25.8%
- **G0:** 16.7%

**TgPKR**
- **G1:** 51.2%
- **S+G2/M:** 22.1%
- **G0:** 26.7%

**TgDNPKR**
- **G1:** 58.5%
- **S+G2/M:** 28%
- **G0:** 13.5%

**PKRKO**
- **G1:** 52.5%
- **S+G2/M:** 36.1%
- **G0:** 11.4%
Figure 5

B

Days in culture

Lin\textsuperscript{c}Kit\textsuperscript{+} cells

C

Days in culture

CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells
Figure 5

D

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<th>TgPKR</th>
<th>WT</th>
<th>TgDNPKR</th>
<th>PKRKO</th>
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<td>WB: Actin</td>
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hPKR, mPKR
Figure 6

D

E

WT

Control

+ PKRI

TgPKR

Control

Δ + PKRI

Growth Factors

Colonies Formation (relative %)

F

G

TgDNPKR

Control

Δ + PKRI

Colony Formation (relative %)

Growth Factors

TgPKR

PKRKO

Control

Δ + PKRI

Colony Formation (relative %)

Growth Factors
Figure 6

H

Colony Formation (relative %)

WT
TgPKR
TgDNPKR
PKRKO

IFN gamma (IU/ml)

I

Colony Formation (relative %)

WT
TgPKR
TgDNPKR
PKRKO

TNF alpha (ng/ml)

J

Survival of clonogenic cells

WT
TgPKR
TgDNPKR
PKRKO

Irradiation dose (Gy)
PKR regulates proliferation, differentiation and survival of murine hematopoietic stem/progenitor cells

Xiangfei Liu, Richard L. Bennett, Xiaodong Cheng, Michael Byrne, Mary K. Reinhard and W. Stratford May Jr.