Published in final edited form as:

Exp Hematol. 2015 March ; 43(3): 243–252.e1. doi:10.1016/j.exphem.2014.10.014.

Ryk receptor regulates hematopoietic stem and progenitor sensitivity to myelosuppressive injury in mice

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Abstract

Maintaining a careful balance between quiescence and proliferation of hematopoietic stem and progenitor cells (HSPCs) is necessary for lifelong blood formation. Previously, we demonstrated that the Wnt5a ligand inhibits HSPC proliferation through a functional interaction with a non-canonical Wnt ligand receptor termed Ryk. Expression of Ryk on HSPCs *in vivo* is associated with a lower rate of proliferation and following treatment with fluorouracil (5-FU), the percentage of Ryk^{+/high} HSPCs increased while the percent of Ryk^{-/low} HSPCs decreased. Based on these data, we hypothesized that one function of the Ryk receptor is to protect HSPCs from the effects of myeloablative agents. We found that Ryk expression on HSPCs is associated with lower rates of apoptosis following 5-FU and radiation. Transient inhibition of Ryk signaling *in vivo* resulted in increased HSC proliferation and decreased HSC function in bone marrow transplant assays. Furthermore, inhibition of Ryk signaling sensitized HSPCs to 5-FU treatment in association with increased levels of reactive oxygen species. Together, these results demonstrated an association between Ryk expression and survival of HSPCs following suppressive injury.

Keywords

Hematopoietic Stem Cells; Receptors; Wnt; Fluorouracil

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Introduction

In adult mammals, the hematopoietic stem cell (HSCs) governs life-long regeneration of mature blood cells (hematopoiesis). HSCs are a rare population of cells that reside in the bone marrow and unique among hematopoietic cells, exhibit the capacity for self-renewal and multi-lineage differentiation [1]. In order to maintain a population of functional HSCs during life, several cellular mechanisms exist to prevent their premature exhaustion. During homeostasis the majority of HSCs are quiescent, in contrast to the more-differentiated hematopoietic progenitor populations that are highly proliferative[2]. Mitotic quiescence protects HSCs against premature senescence as multiple studies have demonstrated that enforced proliferation of HSCs results in decreased self-renewal and eventual loss of function[3].

The quiescent state has also been hypothesized to protect HSCs from cytotoxic insults, such as chemotherapy drugs, that preferentially eliminate actively dividing cells. Since these agents result in considerable reduction in bone marrow cellularity (myelosuppression), HSC survival is required for restoration of normal hematopoiesis. Enhancing the ability of HSCs to withstand such insults results in accelerated recovery of hematopoiesis [4, 5]. Thus, understanding the mechanisms that regulate HSC proliferation and the related ability to survive cytotoxic injury is essential for developing novel approaches for rescuing hematopoiesis following myelosuppression.

In a separate series of studies, we have identified a novel functional role for the Related to Receptor Tyrosine Kinase (Ryk) protein in the regulation of HSC proliferation[6]. Ryk is a Wnt ligand receptor that is expressed in multiple cell types, including HSCs and its expression is associated with decreased HSC proliferation[7–10]. We reported that Ryk functionally interacts with the Wnt5a ligand to maintain quiescence and suppress cell proliferation and the levels of reactive oxygen species (ROS) in HSCs. The connections between Ryk, ROS, and proliferation may be critical for HSC biology as maintaining low levels of ROS in HSCs is necessary for preserving HSC quiescence and long-term function[11, 12].

In addition to their role in HSC proliferation, ROS also regulate the sensitivity of HSCs to myeloablative agents such as fluorouracil (5-FU) and ionizing radiation. Previously, we found that Ryk^{+/high} HSCs have lower ROS levels *in vivo* compared to Ryk^{-/low} HSCs and that the percentage of Ryk^{+/high} HSCs and progenitors (HSPCs) increases following 5-FU[6]. The data formed the basis for our hypothesis that Ryk signaling acts to functionally protect HSCs from the effects of myeloablative injury.

In this study, we found that expression of Ryk is associated with lower rates of apoptosis following both 5-FU and total body irradiation (TBI). To determine a functional role for Ryk in HSPC survival following injury, we treated mice with a neutralizing anti-Ryk antibody. We found that inhibition of Ryk increased steady-state proliferation of HSCs and progenitors and altered their function. Transient inhibition of Ryk decreased HSPC survival following 5-FU treatment in association with increased production of ROS. Together, these data suggest a model in which inhibition of the Ryk receptor results in increased proliferation and

generation of ROS in HSPCs, enhancing their sensitivity to myeloablative agents such as chemotherapy or TBI.

Materials and Methods

Mice—B6.SJL-PtprcaPepcb/BoyJ and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). C57BL/6-Tg (UBC-GFP) 30Scha/J mice were provided by Dr. James Thompson (Roswell Park Cancer Institute, Buffalo, NY)[13]. All mice were housed in sterile conditions in the Roswell Park Cancer Institute facility. For specific experiments, mice were injected with 150 mg/kg 5-fluorouracil intraperitoneally (Sigma Aldrich, St. Louis, MO, USA) and/or 5 mg/kg neutralizing anti-Ryk antibody (RB1491, Abgent, San Diego, CA, USA) or rabbit IgG isotype control intravenously (R and D Systems, Minneapolis, MN, USA). For radiation experiments, mice received 3.0 or 6.0 Gy from a cesium radiation source.

Western blot analysis

Whole cell lysate was prepared using NP-40 buffer (50mM HEPES-KOH, pH7.5, 1% Nonidet P-40, 150mM NaCl, 1mM ethylenediaminetetraacetic acid, 1mM dithiothreitol and a cocktail of protease inhibitors). 15 µg of protein was electrophoresed using a NuPAGE® Novex® 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA USA), transferred to a nitrocellulose membrane and incubated overnight with anti-Ryk antibody (Abgent) at 1:1000 dilution followed secondary antibody (GE Healthcare Life Sciences, Piscataway, NJ USA) at 1:3000 dilution for 1h. As a loading control β-actin antibody (MP Biomedicals, Santa Ana, CA USA) at 1:10,000 dilution was used.

Flow cytometry analysis of bone marrow cells—Central and endosteal mouse bone marrow cells were obtained using the standard protocol adapted from Grassinger, *et al* [14]. Cells were stained with FITC-conjugated anti-mouse lineage markers (CD4 (clone GK1.5), CD8a (53-6.7), B220 (RA3-6B2), Gr-1 (RB6-8C5), CD11b (M1/70), Ter119 (Ter-119), PE-Cy7-conjugated anti-Sca-1 (D7), APC-eFluor780-conjugated anti-C-kit (2B8), PE-conjugated anti-CD150 (mShad), PerCp-Cy5.5 anti-CD48 (93) and biotinylated anti-Ryk (R and D Systems) and incubated on ice for 20 minutes. All antibodies except for anti-Ryk were from eBioscience (San Diego, CA, USA). Cells were subsequently stained with eFluor710-conjugated streptavidin. For this and subsequent experiments, live cells were determined by staining cells with 4',6-diamidino-2-phenylindole (DAPI); live cells were defined as DAPI-negative.

Apoptosis—Bone marrow cells were stained as described with the exception that APC-conjugated anti-lineage markers were used. Cells were stained for active caspase-3 using the FITC Active Caspase-3 Apoptosis Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

Cell Cycle—Bone marrow cells were analyzed for cell cycle status as described [2, 6]. Cell cycle phases were determined by staining cells with Hoechst 33343 (Sigma Aldrich) and APC-conjugated anti-Ki-67 (SolA15,eBioscience).

Reactive oxygen species—Bone marrow cells were stained as described with the exception that APC-conjugated anti-lineage markers were used. Samples were stained with 5.0 μM β -M chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCF) (Invitrogen) at 37°C for 30 minutes and immediately analyzed by flow cytometry[6].

Hematopoietic repopulation assay— 2×10^6 whole bone marrow cells were harvested from antibody-treated C57BL/6 mice and injected retro-orbitally into lethally irradiated (10.5 cGy) B6.SJL-PtprcaPepcb/BoyJ mice along with 2×10^6 C57BL/6-Tg (UBC-GFP) 30Scha/J whole bone marrow cells.

To measure hematopoietic repopulation, peripheral blood cells were harvested 4 and 16 weeks post-transplant and stained with APC-conjugated anti-CD3 (1452C11), PerCp-Cy5.5-conjugated anti-B220, APC-eFluor780 conjugated anti-Gr-1, PE-Cy7 conjugated anti-CD11b, Pacific Blue conjugated anti-CD45.1 (A20), Alexa Fluor 700- conjugated anti-CD45.2 (104). All antibodies were from eBioscience. Engraftment of transplanted cells was defined as greater than 1% of total nucleated live blood cells.

Data analysis and Statistics

Flow cytometry was analyzed using FlowJo version 9.5 (TreeStar, Ashland, OR, USA). For all experiments p-values were determined using unpaired or paired *t*-test.

Results

Ryk+ HSPCs exhibit decreased apoptosis following myeloablative injury

Previously, we observed an increase in Ryk+ HSPCs following treatment with 5-FU that could be due to increased proliferation or survival of Ryk+ HSPCs[6]. Since we found that Ryk+ HSPCs were less proliferative following 5-FU, this suggested that the increased numbers of Ryk+ HSPCs was due to increased survival. To test this, we treated mice with 150 mg/kg of 5-FU and analyzed HSPC apoptosis 48 hours later, using activated caspase-3 as a marker. Because 5-FU down-regulates c-kit, thereby eliminating its use as a functional marker for discriminating HSPC populations, we defined an HSPC population as lineage-negative (lin-), Sca-1+ (LS), CD150+, and CD48-/lo (Figure 1A)[15–17]. In untreated mice, there was no difference in apoptosis between Ryk+ and Ryk- LS, CD150+, CD48-/lo HSPCs (Figure 1B). Following 5-FU treatment, the percentage of apoptotic Ryk+ LS, CD150+, CD48-/lo HSPCs was 4-fold lower than Ryk- cells ($p < 0.01$), suggesting that Ryk may functionally protect progenitors against the apoptosis-inducing effects of 5-FU treatment.

To determine if this effect was specific for 5-FU, we treated mice with 3.0 or 6.0 Gy of total body irradiation (TBI) and analyzed bone marrow 3 days post-irradiation. For this experiment, we analyzed apoptosis in HSPCs using the Lin-, Sca-1+, c-kit+ (LSK), CD150+, CD48-/lo immunophenotype. Following 3.0 Gy of TBI, there was a 1.8-fold increase in the percentage of Ryk- HSCs positive for active caspase-3+ compared to Ryk+ HSPCs ($p < 0.005$, Figure 1C). We observed similar effects following 6.0 Gy of TBI. Together, these data indicate that expression of Ryk is associated with increased survival of HSPCs following myeloablative injury.

Inhibition of Ryk signaling *in vivo* promotes HSPC proliferation

We tested the effect of transient inhibition of Ryk on HSPC function using an anti-Ryk neutralizing antibody that recognizes the Ryk protein in mouse lineage-negative HSPCs (Figure 2A) [18]. Mice were treated with 5.0 mg/kg of or rabbit isotype IgG control for two consecutive days. Twenty-four hours after the second dose, bone marrow cells were harvested and analyzed.

Previously, we found that Ryk⁺ HSPCs exhibited a decreased rate of proliferation compared to Ryk[−] HSPCs. Based on the link between cell proliferation and sensitivity to 5-FU, we tested whether inhibition of Ryk activity altered HSPC proliferation. We treated mice with α -Ryk or isotype control according to the same schedule described earlier and analyzed HSPC populations for changes in cell cycle status by flow cytometry. The immunophenotypes for each population were defined based on Oguro, *et al* [19]. HSCs were defined by the Lin[−], c-kit⁺, Sca-1⁺ (LSK), CD150⁺, CD48^{−/lo} phenotype and multipotent progenitors (MPP) by the LSK, CD150[−], CD48^{−/lo} phenotype. Treatment with α -Ryk resulted in a significant increase in the frequency of total bone marrow cells that exhibited the HSC immunophenotype (Figure 2B, $p < 0.01$). We also observed significant increases in the percentages of two distinct populations of lineage-restricted hematopoietic progenitor cells: HPC-1 (LSK, CD150[−], CD48⁺) and HPC-2 (LSK, CD150⁺, CD48⁺) following treatment with α -Ryk compared to control ($p < 0.01$). In contrast, there was no difference in the percentage of total bone marrow cells with the MPP phenotype.

Based on these data, we expected to detect increased percentages of cells in the active phases of the cell cycle. As predicted, treatment with α -Ryk resulted in a decrease in the percentage of quiescent G₀ HSCs ($p < 0.001$) and an increase in the percentage of HSCs in G₁ compared to control ($p < 0.01$; Figure 2C and 2D). There was no difference in the percentage of HSCs in S/G₂/M phases. Surprisingly, we observed that α -Ryk treatment had a similar effect on the cell cycle distribution of MPPs and also increased the percentage of MPPs in the S/G₂/M phases by 2-fold ($p < 0.05$) compared to control. These effects were also observed in the HPC-1 population. However, the cell cycle status of the HPC-2 population was unaffected. These data suggest that inhibition of Ryk promotes proliferation of HSCs and progenitors *in vivo*. However, the discordant results between cell numbers and cell cycle status for the MPP and HPC-2 populations also suggests that the effects of Ryk inhibition were not restricted to proliferation.

Effect of inhibiting Ryk on HSPC function

To further test whether disruptions in Ryk activity can affect HSPC function, we treated C57BL/6 (CD45.2) mice with α -Ryk or rabbit IgG using the same schedule as described. There was no difference between treatment groups in either bone marrow cellularity or in the ability of bone marrow cells form hematopoietic colonies between groups (Supplemental Figure 1).

To test the ability of bone marrow cells from treated animals to reconstitute hematopoiesis, we transplanted 2×10^6 whole bone marrow cells from treated animals along with bone marrow cells from C57BL/6-Tg (UBC-GFP) 30Scha/J transgenic mice (GFP⁺) at a 1:1 ratio

into lethally irradiated B6.SJL (CD45.1+) recipients. The relative contributions of donor HSPCs to hematopoietic reconstitution were measured through serial analysis of peripheral blood 4 (short-term) and 16 weeks (long-term) post-transplant. Since α -Ryk treatment significantly increased percentage of HSCs within the whole bone marrow (see Figure 2B), we normalized the repopulation data to account for the transplanted number of phenotypic HSCs. We found no significant difference in the short-term repopulating potential of α -Ryk treated bone marrow (Figure 3A). However, α -Ryk treatment significantly decreased long-term repopulation by transplanted HSCs compared to control ($p < 0.05$). In agreement with our previous study, α -Ryk treatment had no effect on reconstitution of myeloid *versus* lymphoid lineages compared to control (Figure 3B)[6]. These data suggest that at the dosage schedule used for these experiments the effects of inhibiting Ryk signaling significantly decrease long-term HSC function.

Inhibition of Ryk signaling *in vivo* promotes HSPC apoptosis following 5-FU treatment

Based on these data, we tested whether transient inhibition of Ryk activity would result increased sensitivity to myeloablative agents. Mice were treated with 5.0 mg/kg α -Ryk or rabbit isotype IgG control for two consecutive days prior to administration of 5-FU. There as no difference between treatment groups on overall bone marrow cellularity (Supplemental Figure 1). Forty-eight hours later, there were a significantly higher percentage of activated caspase-3+ LS CD150+ CD48– HSPCs following α -Ryk treatment compared to control ($p < 0.05$; Figure 4A and 4B). We also detected a significantly higher percentage of apoptotic more mature progenitors (LS CD150– CD48–/lo) following α -Ryk treatment compared to control ($p < 0.05$). There was no difference in the cell cycle distribution of HSCs and MPPs between experimental groups (Figure 4C and 4D).

Inhibition of Ryk signaling *in vivo* increases ROS levels in HSPCs following 5-FU treatment

Several factors contribute to the sensitivity of HSPCs to 5-fluorouracil, including generation of reactive oxygen species (ROS) [20, 21]. These studies, in combination with our observation that Ryk+ HSPCs had lower levels of ROS than their Ryk– counterparts, suggests that decreased ROS levels could contribute to the relative survival of Ryk+ HSPCs following 5-FU treatment [6]. Thus, we tested whether pre-treatment with α -Ryk resulted in increased ROS levels in HSPCs following 5-FU treatment (Figure 5A). Treatment with α -Ryk in combination with 5-FU increased the level of ROS in LS CD150+ CD48–/lo cells by 1.7-fold compared to untreated control mice ($p < 0.01$, Figure 5B). There was no difference in ROS levels in LS, CD150–, CD48–/lo progenitors. This suggests that the increased levels of ROS observed after the treatment combination of α -Ryk and 5-FU could contribute to the increased number of apoptotic LS CD150+ CD48–/lo cells.

Ryk+ HSPCs preferentially reside in the endosteal region of the bone marrow

Recent studies have demonstrated that quiescent HSCs that associate with arterioles within the endosteal region of the bone marrow preferentially survive 5-FU administration[22]. To test whether Ryk+ HSPCs are similarly enriched within the endosteal region, we isolated bone marrow fractions from the central *versus* endosteal regions and analyzed Ryk expression in HSCs and MPPs using the same immunophenotypes as described in Figure 2 (Figure 6A)[14, 23]. In agreement with our previous study, there was a significant increase

in the percentage of Ryk+ cells among the HSC population compared to the more differentiated MPP population in both the central and endosteal fractions (Figures 6B and 6C)[6]. We observed a significant increase in the percentage of Ryk+ HSCs and MPPs in the endosteal fraction compared to the central fraction ($p < 0.001$; Figure 6C), suggesting a preferential localization of Ryk+ HSPCs to distinct regions of the bone marrow.

Discussion

The family of Wnt signaling pathways comprises a complex network that regulates multiple processes in HSCs. We and others have demonstrated that non-canonical Wnt signaling pathways, which do not utilize β -catenin as a secondary messenger, are important regulators of HSC proliferation, self-renewal, and aging[6, 24–26]. Previously, we demonstrated that the Wnt5a ligand, which activates non-canonical Wnt signaling, suppressed proliferation of HSCs and maintains them in a quiescent state[24]. We found that this effect was mediated by the Ryk protein, which can act as either an independent Wnt ligand receptor or as a co-receptor with other Wnt receptors[6]. While these data suggested that Ryk function linked Wnt5a to HSC quiescence and proliferation, the role of Ryk in hematopoiesis remains unclear. In this study, we expand upon our previous work to find a role for Ryk activity in protecting HSPCs from myeloablative stress. Together, our data suggest a model in which Ryk function contributes to the protection of HSCs and progenitors in part through maintaining a low-proliferative state in association with decreased levels of ROS.

One prediction of this model is that Ryk+ HSCs and progenitors would exhibit relatively decreased sensitivity to myeloablative agents that preferentially target actively cycling cells or require a threshold level of ROS to have an effect. In keeping with this prediction, we observed decreased percentage of Ryk+ HSCs and progenitors undergoing apoptosis following treatment with 5-FU or total body irradiation. This difference is only present following injury, suggesting a protective role for Ryk during myeloablative stress.

Our finding that inhibition of Ryk function *in vivo* significantly decreases the percentage of quiescent HSCs and progenitors points to Ryk having a functional role in maintenance of steady-state proliferation. These observations are in agreement with our previous study demonstrating that increased expression of Ryk associated with decreased proliferation[6]. Further support for this model comes from de Graaf, *et al.*, and Forsberg, *et al.*, who observed that decreased Ryk expression was accompanied by increased HSC proliferation[9, 10].

It is possible that Ryk regulates other aspects of HSPC biology besides proliferation such as differentiation. Our finding that treatment with an anti-Ryk antibody reduced long-term repopulating ability of HSCs on a per cell basis is similar to our previous observation that treatment of HSCs *in vitro* with anti-Ryk was sufficient to reduce the frequency of long-term engraftment and repopulation[6]. It is unlikely that the increase in proliferation observed in our study would be sufficient to affect HSC function. Our observation that inhibiting Ryk did not alter clonogenic potential or short-term repopulation suggests a preferential effect on the HSC population, which is in agreement with our previous data showing that Ryk levels decrease as HSCs differentiate. The HSC and MPP populations as defined in this study can

be further separated based on CD229 and CD244 levels and thus it is possible that inhibiting Ryk affects a specific subset of HSCs or MPPs [19].

The ability of Ryk to regulate proliferation of HSPCs may be linked to its role in mediating survival of HSPCs following myeloablative stress. Our observation that inhibition of Ryk results in decreased survival of HSPCs following 5-FU treatment is in agreement with prior studies demonstrating that the relative sensitivity of these cells to 5-FU is connected to their proliferation status[5, 27]. However, we also observed that Ryk+ cells have equivalent relative resistance to 5-FU and TBI. Since 5-FU is relatively more effective on cycling HSCs, while TBI appears to have equal effects on quiescent and cycling HSCs, we hypothesize that additional factors contribute to the increased sensitivity of HSPCs to 5-FU following inhibition of Ryk[28]. This hypothesis is supported by our data indicating that inhibition of Ryk increased apoptosis, but did not affect cell cycle status, following treatment with 5-FU, further suggesting the presence of proliferation-independent mechanisms.

One such factor suggested by our data is that inhibition of Ryk promotes sensitivity to 5-FU by increasing the level of ROS[20, 21]. Our observation that inhibiting Ryk had a preferential effect on ROS levels in less differentiated HSPCs may be due to the increased percentage of Ryk+ cells among the less-differentiated HSPC populations. While increasing ROS levels may contribute to increased sensitivity of HSPCs following Ryk inhibition, additional factors likely contribute to this outcome. This possibility is supported by our observation that while inhibiting Ryk significantly increased ROS levels compared to control, the effect was modest compared to the control IgG cohort.

One explanation for our data showing that Ryk+ HSPCs are preferentially found in the endosteal region while exhibiting decreased ROS levels could be that these cells exhibit a phenotype associated with a more hypoxic state [6, 23, 29]. This association may be independent of local oxygen tension since the endosteal region has been found to be less hypoxic relative to the rest of the bone marrow and HSCs displaying a hypoxic profile are found throughout the bone marrow [30, 31]. Support for this hypothesis comes from our observation that Wnt5a suppressed ROS levels in HSCs *in vitro* through a functional interaction with Ryk[6]. Further work is necessary to test whether Ryk+ HSPCs are associated with arteriole niches within the endosteal region that confer protection against myeloablative injury [22].

The molecular links between Ryk and downstream effects on cell proliferation and survival are unclear. Ryk can signal through canonical or non-canonical pathways depending on the cellular context[8, 32–34]. Although Ryk can regulate Wnt5a-mediated non-canonical Wnt signaling in HSCs, canonical Wnt signaling is increased in HSCs following 5-FU treatment [25, 35]. Thus, it is possible that Ryk regulates distinct pathways depending on the state of hematopoiesis. There is precedence for this reprogramming with regards to Wnt signaling as splice variants in focal adhesion kinase found in patients with AML cause Wnt5a-mediated activation of the canonical Wnt pathway[36].

Although our *in vivo* data mirror our previous *in vitro* results, we cannot rule out the possibility that the anti-Ryk antibody opsonizes cells, targeting them for degradation. Thus, this process could also contribute to the effects of anti-Ryk across the different HSPC populations. The use of Fab fragments could resolve this issue. Ultimately, the use of genetic loss-of-function models will be required to further elucidate the cellular and molecular mechanisms of Ryk signaling in hematopoiesis[7].

These data support a model in which Ryk protects HSPCs from a range of myeloablative stresses, potentially through regulating proliferation and ROS levels. Together, they illustrate a clinical potential for manipulating Ryk signaling in HSPCs in the myeloablative or myelosuppressive setting, such as chemotherapy for treatment of solid tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank the members of the Laboratory Animal and Flow Cytometry Resources at Roswell Park Cancer Institute, which are supported by Cancer Center Support Grant P30 CA016056, for technical assistance during these experiments. Support for these studies was provided by the National Blood Foundation, Cancer Center Support Grant Developmental Funds, and by institutional funds provided by Roswell Park Cancer Institute.

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- HSPCs that express Ryk exhibit decreased apoptosis following 5-FU and radiation
- Inhibition of Ryk increases proliferation of HSPCs
- Inhibition of Ryk decreases repopulating ability of HSPCs
- Following 5-FU treatment, inhibition of Ryk increases HSPC apoptosis

Figure 1. Effect of myelosuppressive injury on Ryk-expressing HSPCs

Mice were injected with 150 mg/kg of 5-fluorouracil (5-FU), irradiated with 3.0 or 6.0 Gy TBI or untreated and their bone marrow cells analyzed by flow cytometry 2 (5-FU) or 3 (irradiation) days later. (A) Representative flow cytometry analysis of Ryk in lineage negative (Lin-), Sca-1+ (LS), CD150+, CD48- cells. Expression of CD150 and CD48 in Lin-, Sca-1+ (LS) cells (middle) was defined based on isotype controls (left). (B) Representative flow cytometry analysis of apoptosis in LS, CD150+, CD48- cells from 5-FU treated mice. Apoptosis of Ryk+ and Ryk- cells (middle and right) was determined

using activated caspase-3 as a marker (isotype control, left). (C) Average percent of activated caspase-3+ Ryk+ and Ryk- LS, CD150+, CD48- cells in untreated (n = 4, left) and 5-FU treated mice (n = 4, right). (C) Average percentage of activated caspase-3+ Ryk+ and Ryk- LS, CD150+, CD48- cells in mice irradiated with 3.0 Gy (n = 5, left) or 6 Gy (n = 5, right) total body irradiation. Error bars represent standard deviation. P-values were generated using paired *t*-tests.

Figure 2. Effect of Ryk inhibition on HSPC Proliferation

C57BL/6 mice were treated with 5mg/kg blocking Ryk antibody (α-Ryk) or isotype IgG (control) on two consecutive days. (A) Representative Western Blot analysis of Ryk protein levels (67.5 kDa) in 32D cells (negative control, left) and lineage-negative cells (right). Detection of actin protein was used as a loading control. (B) Average percentage of hematopoietic stem cells (HSC: LSK CD150+, CD48-), multipotent progenitors (MPP: LSK, CD150-, CD48-); hematopoietic progenitor cell-1 (HPC-1: LSK, CD150-, CD48+), and hematopoietic progenitor cell-2 (HPC-2: CD150+, CD48+) among total bone marrow cells from control (black) or α-Ryk treated (gray) mice (n = 3). (C) Representative flow cytometry analysis of the cell cycle in the HSC population. Top: Determination of G0/G1 and S/G2/M phases in HSCs from control (left) *versus* α-Ryk (right) treated mice using Hoescht dye. Bottom: Analysis of G0 and G1 phases in HSCs from control (left) *versus* α-Ryk (right) treated mice using Ki-67. G0 cells were defined as Ki-67 negative. Numbers indicate the percentage of G0 or G1 cells among total G0 + G1 cells. (D) Average percentage of HSCs (top left), MPPs (top right), HPC-1 (bottom left) and HPC-2 (bottom right) in each cell cycle phase in control (black bar) *versus* α-Ryk (gray bar) treated mice (n = 3). Error bars represent standard deviation. P-values were generated using unpaired *t*-tests.

Figure 3. Effect of Ryk inhibition on HSPC function

C57BL/6 mice (CD45.2+) were treated with 5mg/kg blocking Ryk antibody (α-Ryk) or isotype IgG (control) on two consecutive days. Bone marrow cells from 3 pooled α-Ryk or control treated mice were transplanted into B6.SJL mice with GFP+ bone marrow at a 1:1 ratio, and repopulation was measured through peripheral blood analysis. (A) Hematopoietic chimerism of peripheral blood cells 4 (top) and 16 weeks (bottom) after transplant. Chimerism is normalized to input HSC number (control, 234; α-Ryk, 320). Each point represents a single recipient (control, n = 5; α-Ryk n = 4 surviving recipients (out of 5

transplanted mice). Average percent contribution by donor cells (% CD45.2+, GFP–) is marked with a horizontal bar. (B) Average percentage of lymphoid (black bar) or myeloid (gray bar) CD45.2+, GFP– peripheral blood cells in recipients 16 weeks after transplantation. Myeloid cells are positive for the cell surface markers Gr-1 and/or CD11b. Lymphoid cells are positive for the cell surface markers CD3 or B220. For all panels, error bars represent standard deviation and P values between control *versus* α -Ryk groups were determined using unpaired *t*-test.

Figure 4. Effect of inhibiting Ryk on HSPC apoptosis and proliferation following 5-FU treatment C57BL/6 mice (CD45.2+) were treated with 5 mg/kg anti-Ryk antibody (α -Ryk) or isotype IgG (control) on two consecutive days. Twenty-four hours later, mice were injected with 150 mg/kg 5-FU and their bone marrow cells analyzed 2 days later. (A) Representative flow cytometry analysis of apoptosis in Lin-, Sca-1+ (LS), CD150+, CD48- cells following 5-FU administration in mice treated with rabbit IgG isotype control antibody (left) or α -Ryk antibody (right). Gates were drawn based on isotype controls. (B) Average percentage of activated caspase-3+ Lin-, Sca-1+ (LS), CD150+, CD48- and CD150-, CD48- cells following 5-FU administration in mice treated with rabbit IgG isotype control antibody (black) or α -Ryk antibody (gray) ($n = 6$). (C) Representative flow cytometry analysis of the cell cycle in LS, CD150+, CD48- cells using Hoescht and Ki-67. Top: Determination of G₀/G₁ and S/G₂/M phases in cells from control (left) *versus* α -Ryk (right) treated mice following 5-FU treatment. Bottom: Analysis of G₀ and G₁ phases in HSCs from control (left) *versus* α -Ryk (right) treated mice following 5-FU treatment. G₀ cells were defined as Ki-67 negative. Numbers indicate the percentage of G₀ or G₁ cells among total G₀ + G₁

cells. (D). Average percentage of LS, CD150+, CD48– (left) and LS, CD150–, CD48– cells (right) in each cell cycle phase following 5-FU administration in control (black) *versus* α -Ryk (gray) treated mice (n = 3). Error bars represent standard deviation. P-values were generated using unpaired *t*-tests.

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Figure 5. Effect of inhibiting Ryk on ROS levels in HSPCs following 5-FU treatment

C57BL/6 mice were pre-treated with isotype or anti-Ryk antibody (α-Ryk) prior to administration of 5-FU and stained with chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCF) to detect ROS. (A) Representative flow cytometry analysis of ROS levels in bone marrow cells with the HSC immunophenotype (defined as lin⁻, Sca-1⁺, CD150⁺, CD48⁻). ROS levels in HSCs from untreated mice (gray), mice treated with isotype control antibody and 5-FU (dotted line), and mice treated with anti-Ryk and 5-FU (solid line) are displayed. (B) Average median fluorescence intensity (MFI) of the DCF signal in HSCs (black) and MPPs (gray; lin⁻, Sca-1⁺, CD150⁻, CD48⁻) in untreated mice, mice treated with

isotype and 5-FU, and mice treated with α -Ryk and 5-FU (n = 6 per group). Error bars represent standard deviation. P-values were generated using unpaired *t*-tests.

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Figure 6. Analysis of Ryk levels in central versus endosteal HSPCs

(A) Representative flow cytometry analysis from central and endosteal mouse bone marrow. Central and endosteal bone marrow fractions were collected and stained for hematopoietic stem and progenitor markers and the Ryk receptor. Whole bone marrow was gated for singlets, live cells (DAPI-), lineage-, c-kit+, and Sca-1+ (LSK cells). HSCs were defined as LSK CD150+ CD48- and MPPs were defined as LSK CD150- CD48- cells. (B) Representative flow cytometry analysis of Ryk levels in HSPCs from central (left) and endosteal (right) HSPC populations. The percentages of Ryk+ and Ryk- MPPs and HSCs

are noted. (C) Average percentage of Ryk+ HSCs and MPPs in central (black bars) and endosteal (grey bars) fractions of bone marrow (n=3). Error bars represent standard deviation. P-values were generated using paired *t*-tests.

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