Rce1 deficiency accelerates the development of a K-RAS-induced myeloproliferative disease

Running Title: Rce1 Deficiency and K-RAS-induced MPD

Annika M. Wahlstrom¹, Briony A. Cutts¹, Christin Karlsson¹, Karin M. E. Andersson¹, Meng Liu¹,², Anna-Karin M. Sjogren¹, Birgitta Swolin³, Stephen G. Young⁴, and Martin O. Bergo¹

From the ¹Wallenberg Laboratory, Department of Medicine, Sahlgrenska University Hospital, S-413 45 Gothenburg, Sweden; the ²Department of Neurosurgery, Qilu Hospital, Shandong University, Jinan, 250012, China; the ³Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital; and the ⁴Department of Internal Medicine, David Geffen School of Medicine, University of California Los Angeles, CA 90095, USA.

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Correspondence: Martin Bergo, Associate Professor of Medicine, Wallenberg Laboratory, Department of Medicine, Sahlgrenska University Hospital, S-413 45 Gothenburg, Sweden. Tel: +46-31-3423832, Fax: +46-31-823762; E-mail: martin.bergo@wlab.gu.se

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AMW  designed and performed research
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CK   analyzed data
KMEA performed research
ML   performed research
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BS   designed research and analyzed data
SGY  designed research
MOB  designed research and wrote the paper
Abstract

The RAS proteins undergo farnesylation of a carboxyl-terminal cysteine (the “C” of the carboxyl-terminal CaaX motif). Following farnesylation, the three amino acids downstream from the farnesylcysteine (the –aaX of the CaaX motif) are released by RAS converting enzyme 1 (RCE1). We previously showed that inactivation of Rce1 in mouse fibroblasts mislocalizes RAS proteins away from the plasma membrane and inhibits RAS transformation. We therefore hypothesized that inactivation of Rce1 might inhibit RAS transformation in vivo. To test this hypothesis, we used Cre/loxP recombination techniques to simultaneously inactivate Rce1 and activate a latent oncogenic K-RAS allele in hematopoietic cells in mice. Normally, activation of the oncogenic K-RAS allele in hematopoietic cells leads to a rapidly progressing and lethal myeloproliferative disease. Contrary to our hypothesis, the inactivation of Rce1 actually increased peripheral leukocytosis, increased the release of immature hematopoietic cells into the circulation and the infiltration of cells into liver and spleen, and caused mice to die more rapidly. Moreover, in the absence of Rce1, splenocytes and bone marrow cells expressing oncogenic K-RAS yielded more and larger colonies when grown in methylcellulose. We conclude that inactivation of Rce1 worsens the myeloproliferative disease caused by an oncogenic K-RAS.
Introduction

Activating mutations in RAS genes result in constitutive signaling of the RAS proteins and are implicated in the pathogenesis of many human cancers, including several hematological malignancies\textsuperscript{1,2}. For example, activating mutations in RAS are present in up to 44% of patients with acute myeloid leukemia\textsuperscript{3}. Hematological malignancies also occur when RAS signaling is increased, as observed in diseases such as neurofibromatosis (NF\textsubscript{1}) where there is a genetic abnormality in a RAS-interacting protein\textsuperscript{4}.

The RAS proteins undergo several posttranslational processing steps, beginning with the farnesylation of the cysteine residue (the “C” of the CaaX motif) by protein farnesyltransferase (FTase). After this “lipidation” step, the last three amino acids of the protein (the –aaX of the CaaX motif) are released by RAS converting enzyme 1 (RCE1), and the newly exposed farnesylcysteine is methylated by isoprenylcysteine carboxyl methyltransferase (ICMT). Several RAS isoforms (but not K-RAS\textsubscript{4B}) are also palmitoylated at nearby cysteine residues\textsuperscript{5}.

One strategy to block RAS-induced oncogenic transformation is to mistarget the RAS proteins within cells by inhibiting the enzymes that carry out the posttranslational modifications of the RAS proteins. Early preclinical trials of FTase inhibitors (FTIs) against RAS-induced tumors demonstrated significant efficacy with low toxicity\textsuperscript{6}. However, in clinical trials of human solid tumors, the FTIs have not met the high expectations, in part because RAS proteins can be isoprenylated by a related isoprenyltransferase, geranylgeranyltransferase type I (GGTase-I), in the presence of an FTI\textsuperscript{7}.

We have evaluated the possibility of inhibiting RCE1 as a strategy to prevent RAS-induced oncogenic transformation. One potential advantage of this strategy is that RCE1 inhibition would interfere with the processing of the RAS proteins regardless of whether they were farnesylated or geranylgeranylated. In addition, this strategy would not be expected to cause
significant toxicity in vivo. Indeed, we have inactivated \textit{Rce1} in the liver, spleen, and bone marrow of mice and not observed significant adverse effects\textsuperscript{8}. Also, Ayiagari et al.\textsuperscript{9} showed that \textit{Rce1}-deficient fetal liver cells are capable of rescuing hematopoiesis in lethally irradiated mice.

Several lines of investigation suggest that inhibition of RCE1 would inhibit RAS-induced oncogenic transformation. First, inactivation of \textit{Rce1} mislocalized the RAS proteins and reduced cell proliferation and the anchorage-independent growth of RAS-transformed fibroblasts in soft agar and nude mice\textsuperscript{8,10}; second, in the absence of \textit{Rce1}, skin carcinoma cells grew slowly and were highly sensitive to the effects of an FTI\textsuperscript{8}; and third, several potent RCE1 inhibitors have been developed\textsuperscript{11,12}, and two of them reduced the anchorage-independent growth of K-RAS-transformed cells \textit{in vitro}\textsuperscript{13,14}. However, nothing is known about the effects of inhibiting RCE1 on the growth of RAS-induced malignancies in vivo.

In this study, we determined if inactivation of \textit{Rce1} in mice would inhibit the development of a K-RAS-induced myeloproliferative disease (MPD) in vivo. To accomplish this, we used \textit{Cre} recombinase to simultaneously inactivate the expression of \textit{Rce1} and activate the expression of oncogenic K-RAS\textsuperscript{G12D} in hematopoietic cells.

\textbf{Materials and Methods}

\textbf{Breeding mice for in vivo experiments}

Mice with a conditional \textit{Rce1} knockout allele (\textit{Rce1}\textsuperscript{fl}) have been described\textsuperscript{8}. Mice with a \textit{Kras2\textsuperscript{LSL}} allele\textsuperscript{15} have an activating mutation (G12D) in the \textit{Kras2} gene and a “floxed” transcriptional terminator sequence upstream in the promoter (LSL; \textit{loxP-STOP-loxP}). \textit{Cre} expression results in the removal of the STOP cassette, which turns on the expression of K-RAS\textsuperscript{G12D}. For our experiments, \textit{Rce1}\textsuperscript{fl/fl} mice with a \textit{Kras2\textsuperscript{LSL}} allele were bred with \textit{Rce1}\textsuperscript{fl/+} mice harboring an interferon-inducible Mx1-\textit{Cre} transgene\textsuperscript{16} to generate
Rce1^{fl/fl}Kras2^{LSL/+}Mx1-Cre mice. In those mice (hereafter designated Rce1^{fl/fl}K^{LSL,M}), Cre expression simultaneously inactivated Rce1 expression and activated the expression of K-RAS^{G12D} in bone marrow cells. These cells are designated Rce1^{Δ/Δ}K^{G12DM}. For experimental controls, we used mice harboring a single mutant Rce1 allele (Rce1^{fl/+}K^{LSL,M} mice); in those mice, Cre inactivates one Rce1 allele and reduces Rce1 expression by 50%. The mice were maintained on a 129/Sv and C57BL/6 mixed genetic background and the Rce1^{fl/fl}K^{LSL,M} Rce1^{fl/+}K^{LSL,M} mice were littermates. Animal procedures were approved by the animal research ethics committee in Gothenburg.

Genotyping

Mice were genotyped by PCR amplification of genomic DNA from tail biopsies. The Rce1^{fl} allele was detected with forward primer 5′–GCTTTTGGAAAGAACAGGGGCC–3′ and reverse primer 5′–CTCACCTCCAGTTGCTGCTCATC–3′. This PCR reaction yields a 350-bp fragment from the Rce1^{fl} allele and a 270-bp fragment from the Rce1^{+} allele. The Mx1-Cre transgene was detected with forward primer 5′–GAGCTCCATTCATGTGTGGT–3′ and reverse primer 5′–CTAGAGCCTGTTTTGCACGTTC–3′; the DNA product was 1009 bp. The Kras2^{LSL} allele was detected with forward primer 5′–CCTTTACAAGCGACGCAGACTGTAGA–3′ and reverse primer 5′–AGCTAGCCACCATGGGTAGTAAGTCTGCA–3′; the amplified fragment was 600 bp. The presence of the activated Kras2^{G12D} allele was detected with forward primer 5′–GGGTAGGTGTTGGATAGCTG–3′ and reverse primer 5′–TCCGAATTCCAGTGACTACAGATGTACAGAG–3′; the amplified fragment was 320 bp.

Quantifying the efficiency of Cre-induced recombination of the Rce1^{fl} allele

DNA was prepared from spleen and bone marrow cells and used for real-time quantitative PCR (QPCR) with Power SYBR® Green PCR Master Mix on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The following primer
pairs were used: \( Rce1, \ 5'\text{--CGAGTAAATCTGTGGGAGAGG--3'} \) and \( 5'\text{--CGGTGCAATAACTTGGTTTC--3'} \); \( Pggt1b \) (used as a control), \( 5'\text{--CATGTCGTGCTGCTTTCATA--3'} \) and \( 5'\text{--CAGTTTACCCATCAGGCACA--3'} \).

**Injection of polyinosinic-polycytidylic acid and monitoring**

The Mx1-Cre transgene was activated with intraperitoneal injections of 400 µg polyinosinic-polycytidylic acid (pI-pC; Sigma) 21 days after birth (at weaning) once every other day for a total of four injections. Blood was drawn from a tail vein before the first pI-pC injection (time point “0”) and once per week for the duration of the experiment. The blood was analyzed with a Hemavet 950FS cell counter (Drew Scientific, Oxford, CT, USA) and by manual differential counts of May-Grünwald-Giemsa–stained smears by two trained observers blinded to genotype (200 white blood cells per slide were evaluated). At 3 and 5 weeks, groups of mice were sacrificed and tissues harvested.

**Histology**

Tissues were fixed in 4% PBS-buffered formalin, dehydrated in 70–100% ethanol, and cleared in xylene. The tissues were embedded in paraffin and 4–5 µm sections were stained with hematoxylin and eosin. Sections were viewed and photographed in a Zeiss Axioplan 2 light microscope. Cytospin preparations of bone marrow cells were stained with May-Grünwald-Giemsa. Cells of the monocytic and granulocytic lineages were identified by staining for nonspecific and specific esterases, respectively, using α-naphthyl butyrate and naphtol AS-D chloroacetate as substrates.

**Colony assays**

Spleen cells (10^5) and bone marrow cells (2 × 10^4) harvested 5 weeks after pI-pC injections of \( Rce1^{fl/fl}K^{LSL-M} \), \( Rce1^{fl/+}K^{LSL-M} \), and wild-type mice were seeded in duplicate wells in methylcellulose medium (MethoCult M3234, StemCell Technologies) in the absence of
growth factors. Ten days later, the number and size of colonies were scored. Genomic DNA from individual colonies was genotyped by PCR (to detect the activated \textit{Kras}^{G12D} allele and the excision of \textit{Rce1}). Cytospin preparations of cells in individual colonies were stained with May-Grünwald-Giemsa and analyzed by light microscopy.

**Fluorescence-activated cell sorting (FACS)**

Peripheral blood mononuclear cells, bone marrow cells, and cells from methylcellulose colonies were incubated with antibodies against cell-surface antigens [CD11b (cat \# 550993), CD14 (cat \# 553739), CD45 (cat \# 557695), and CD117 (cat \# 553355) (Pharmingen)] and analyzed in a FACSaria (BD Biosciences). Data was analyzed with CellQuest software (BD Biosciences).

**Isolation of mouse embryonic fibroblasts and cell proliferation assays**

Mouse embryonic fibroblasts (MEFs) were isolated from \textit{Rce1}^{fl/fl} \textit{K}^{LSL} and \textit{Rce1}^{fl/+} \textit{K}^{LSL} embryos (lacking the Mx1-\textit{Cre} transgene) at embryonic (E) day 13.5–16.5. Experiments were performed on primary (passage 0–3) and on spontaneously immortalized MEFs (passage 15–35). 5 x 10⁵ cells were seeded onto 60-mm dishes in the presence of adenovirus encoding \textit{Cre} or \textit{β}-gal (10⁹ pfu/mL AdRSV\textit{Cre} and AdRSV\textit{lacZ}, respectively; Iowa University, IO, USA). \textit{Cre}-adenovirus treatment of \textit{Rce1}^{fl/fl} \textit{K}^{LSL} cells produced \textit{Rce1}^{Δ/Δ} \textit{K}^{G12D} cells that expressed endogenous K-RAS^{G12D} but lacked \textit{Rce1} expression; \textit{Cre}-adenovirus treatment of \textit{Rce1}^{fl/+} \textit{K}^{LSL} cells produced \textit{Rce1}^{Δ/+} \textit{K}^{G12D} cells that expressed endogenous K-RAS^{G12D} and lacked 50% of \textit{Rce1} expression. 2 x 10⁴ cells of each genotype were then seeded in triplicate wells in 12-well plates. At various time points, cells were trypsinized and counted in a cell counter (NucleoCounter, Chemometec). DNA was extracted from a portion of the cells and the excision of the \textit{Rce1} allele and the appearance of the activated \textit{Kras}^{G12D} allele were assessed by PCR.
Western blots

Tissue pieces (50–150 mg) were lysed in ice-cold buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 0.1% sodium dodecylsulphate, 1% NP-40, 10 mM NaF, 1 mM phenylmethanesulphonyl fluoride, 2 mM orthovanadate, and the Complete Mini protease inhibitor cocktail (Roche)]. The lysates were homogenized, sonicated, and centrifuged at 20,000 × g for 20 min and equal amounts of total protein of the supernatant were size-fractionated on 10–20% sodium dodecylsulfate polyacrylamide gels (Criterion®, Bio-Rad). The proteins were transferred onto nitrocellulose membranes and incubated with antibodies recognizing phosphorylated ERK1/2 (9106), phosphorylated AKT (9271), total ERK (9102, Cell Signaling, Danvers, MA), and p21⁰⁰⁰⁰ (sc-6246, Santa Cruz). Protein bands were visualized with a horseradish peroxidase–conjugated secondary antibody (sc-2314 and sc-2313, Santa Cruz) and the Enhanced Chemiluminescence kit (Amersham).

Statistical analyses

Data are plotted as mean and standard error of the mean (SEM). Differences in the concentrations and percentages of white blood cells, the colony-forming ability of hematopoietic cells, and the proliferation of cells in culture were determined with Student’s t test; survival was assessed by the Mann-Whitney U test.
Results

Inactivation of Rce1 exacerbates K-RAS-induced myeloproliferative disease

To determine if Rce1 deficiency would inhibit the development of MPD, we monitored pI-pC-injected Rce1^{fl/fl}\text{K}{\text{LSLM} mouse and control Rce1^{fl/+}\text{K}{\text{LSLM} mice. The pI-pC-injected Rce1^{fl/+}\text{K}{\text{LSLM mice developed progressive leukocytosis (Figure 1A) with an increased percentage of myeloid cells (supplementary table). The pI-pC-injected Rce1^{fl/+}\text{K}{\text{LSLM mice exhibited an increase in white blood cell counts compared with the Rce1^{fl/+}\text{K}{\text{LSLM mice; this increase was statistically significant 2 weeks after the pI-pC injections (Figure 1A). At 5 weeks, the mean white blood cell count in Rce1^{fl/+}\text{K}{\text{LSLM mice was 106 \pm 12 \times 10^9/L (n = 15) compared with 39 \pm 3 \times 10^9/L (n = 11) in Rce1^{fl/+}\text{K}{\text{LSLM mice (P < 0.0001). The increased proliferation of white blood cells in the pI-pC-injected Rce1^{fl/+}\text{K}{\text{LSLM mice was associated with reduced survival (Figure 1B; P = 0.035). We conclude that Rce1 deficiency accelerates the development of K-RAS^{G12D}-induced MPD and reduces survival.

The K-RAS-induced MPD is associated with accumulation of immature myeloid cells and increased tissue infiltration in the setting of Rce1 deficiency

In the pI-pC-treated control Rce1^{fl/+}\text{K}{\text{LSLM mice with MPD, the percent of immature myeloid cells was relatively minor (11\% vs. 3\% before pI-pC injections) (Figure 2 and supplementary table). In contrast, there was a dramatic increase in the percent of immature myeloid cells in pI-pC-treated Rce1^{fl/+}\text{K}{\text{LSLM mice (Figure 2A and supplementary table). Overall, 58\% of white blood cells in these mice were immature. There was a proportionate reduction in the percentage of lymphocytes in the two groups of mice (Figure 2B). Thus, the increase in white blood cell counts in pI-pC-treated Rce1^{fl/+}\text{K}{\text{LSLM mice relative to control mice with MPD was due to an accumulation of immature myeloid cells.

To determine if the enhanced production of immature white blood cells was associated with increased myeloid infiltration into vital tissues, we carried out histological studies. Five
weeks after pI-pC injections, increased cellularity was noted in the bone marrow, both in 
\( Rce1^{fl/+}K^{LSLM} \) and \( Rce1^{fl/fl}K^{LSLM} \) mice (Figure 3A, B). FACS analyses and double-esterase staining of bone marrow demonstrated an increased proportion of CD45\(^+\)/CD11b\(^+\)/CD14\(^-\) cells expressing specific esterase—which is consistent with a granulocytic expansion. The proportion of bone marrow cells expressing specific esterase increased from 25.0 ± 3.2% in wild-type mice to 50.8 ± 1.5% in the \( Rce1^{fl/+}K^{LSLM} \) mice \((P = 0.007) \) and further increased to 73.5 ± 1.3% in the \( Rce1^{fl/fl}K^{LSLM} \) mice \((P = 0.01 \text{ vs. } Rce1^{fl/+}K^{LSLM} \) mice; \( P = 0.001 \text{ vs. wild-type} \)). The proportion of CD45\(^+\)/CD117\(^+\) bone marrow cells increased from 4.5% in wild-type mice to 8.1% in \( Rce1^{fl/+}K^{LSLM} \) mice and 9.3% in \( Rce1^{fl/fl}K^{LSLM} \) mice.

The \( Rce1^{fl/+}K^{LSLM} \) mice exhibited a mild-to-moderate infiltration of leukocytes into the liver and spleen, effacement of splenic architecture with extramedullary hematopoiesis, and adenoma formation in the lung (Figure 3C, E, G). In the pI-pC-treated \( Rce1^{fl/+}K^{LSLM} \) mice, there was a severe infiltration of leukocytes into the liver with congestion of central veins, swelling of hepatocytes, and areas of necrosis (Figure 3F). In addition, there was a complete effacement of splenic architecture, and adenoma formation with diffuse hyperplasia in the lung (Figure 3D, H)—likely contributing factors in the rapid demise of the \( Rce1^{fl/+}K^{LSLM} \) mice.

To determine the efficiency of Cre-induced recombination in hematopoietic tissues of pI-pC-injected \( Rce1^{fl/+}K^{LSLM} \), we performed quantitative PCR of genomic DNA: the efficiency was 95 ± 1.6% in spleen and 90 ± 2.2% in bone marrow \((n = 3) \). Thus, the majority of the cells in those tissues had inactivated both alleles of \( Rce1 \).

To determine the consequences of inactivating \( Rce1 \) in K-RAS\(^{G12D} \)-expressing tissues on downstream signaling molecules, we performed western blots. There were increased levels of phosphorylated ERK1/2 in the spleens and livers of pI-pC-injected \( Rce1^{fl/+}K^{LSLM} \) and
Rce1^{fl/fl}K^{LSL}-M compared to control mice without MPD (Supplementary Figure). The levels of phosphorylated AKT, p21^{CIP}, and total ERK1/2 was not different.

**Enhanced colony-forming ability of Rce1-deficient K-RAS^{G12D}-expressing hematopoietic cells**

The spleen weights in Rce1^{fl/fl}K^{LSL}-M and Rce1^{fl/+}K^{LSL}-M mice were similar 3 and 5 weeks after pI-pC injections (Figure 4A). However, given the more advanced histological findings in spleens from Rce1^{fl/fl}K^{LSL}-M mice (Figure 3D), we assessed the ability of splenocytes from the different mice to form colonies in methylcellulose. Rce1^{fl/+}K^{LSL}-M splenocytes were capable of forming colonies in the absence of growth factors, although the colonies were quite small (mean number of colonies per 10^5 cells, 9 ± 3; mean size, 0.19 ± 0.02 mm, Figure 4B, C). In the absence of Rce1, there was a 6.6-fold increase in the number of colonies and a 3.4-fold increase in colony size (Figure 4B, C). With bone marrow cells, there was a 2-fold increase in colony number (Figure 4D). Hematopoietic cells from wild-type mice did not form colonies in the absence of growth factors. The splenocyte colonies from both Rce1^{fl/+}K^{LSL}-M and Rce1^{fl/fl}K^{LSL}-M spleens were composed of CD45+/CD11b+/CD13+/CD14− cells that morphologically resembled macrophages (Figure 4E). PCR amplification of genomic DNA from individual colonies demonstrated that the “floxed” Rce1 gene was excised and the Kras2^{G12D} allele was activated (Figure 4F).

**Rce1 deficiency does not enhance the proliferation of K-RAS^{G12D}-expressing fibroblasts**

To determine the impact of Rce1 deficiency on the proliferation of other cell types expressing K-RAS^{G12D}, we isolated Rce1^{fl/fl}K^{LSL} and control Rce1^{fl/+}K^{LSL} MEFs and treated them with a Cre-adenovirus. As expected, Cre-adenovirus treatment of Rce1^{fl/+}K^{LSL} MEFs activated the expression of K-RAS^{G12D} and increased cell proliferation (Figure 5A). In experiments with both primary and spontaneously immortalized cell lines, the complete inactivation of Rce1 in Rce1^{fl/fl}K^{LSL} MEFs attenuated the increased proliferation caused by K-RAS^{G12D}. Thus, the
inactivation of *Rce1* reduced the proliferation of K-RAS<sup>G12D</sup>-expressing MEFs, even though the same genetic intervention increased the proliferation of K-RAS<sup>G12D</sup>-expressing hematopoietic cells.

**Discussion**

We suspected that inactivation of *Rce1* would inhibit the development of a K-RAS-induced MPD *in vivo*. This suspicion was not upheld. To our surprise, the inactivation of *Rce1* accelerated the development of MPD, increased the release of immature and dysplastic cells into the circulation, and caused mice to die more rapidly, probably because of infiltration and proliferation of cells in vital tissues. These studies suggest that RCE1 inhibitors would not be useful for the treatment of RAS-induced hematological malignancies. Indeed, such a strategy could be harmful.

Our experiments involved the use of *Cre* recombinase to simultaneously inactivate *Rce1* and activate K-RAS<sup>G12D</sup>. This strategy worked as planned. PCR analyses of genomic DNA from multiple individual splenocyte colonies confirmed that *Rce1* had been fully deleted and that the *Kras2*<sup>G12D</sup> allele was activated. Moreover, *Rce1* was deleted in more than 90% of the cells in spleen and bone marrow as judged by quantitative PCR.

Activation of the K-RAS<sup>G12D</sup> allele led to a rapidly progressing MPD, with splenomegaly, increased numbers of mature granulocytes in the circulation, and infiltration of cells in liver and spleen. However, fewer than 20% of the myeloid cells in the blood were immature, so we cannot claim that the mice developed an acute leukemia syndrome. These findings are entirely consistent with earlier studies on the activation of the latent K-RAS<sup>G12D</sup> allele in hematopoietic cells by Braun *et al.*<sup>17</sup> and Chan *et al.*<sup>18,19</sup>. In the setting of *Rce1* deficiency, the K-RAS<sup>G12D</sup>-induced MPD progressed into an accelerated-phase MPD with a maturation arrest evident by the more severe leukocytosis and the dramatic increase in the number of immature myeloid cells in the blood. Also, *Rce1*-deficient hematopoietic cells displayed an
increased capacity to grow in methylcellulose in the absence of growth factors. However, even in the setting of $Rce1$ deficiency, a *bona fide* acute leukemia did not occur, as the number of myeloblasts in the blood remained lower than 20%.

The finding of increased numbers of white blood cells in the absence of $Rce1$ expression is consistent with data reported a few years ago by Aiyagari and coworkers. To define the functional importance of $Rce1$ in normal hematopoiesis, they compared the ability of $Rce1^{-/-}$ and wild-type fetal liver cells to rescue hematopoiesis in lethally irradiated mice. The mice that were replenished with $Rce1^{-/-}$ cells actually had higher white blood cell counts than mice receiving $Rce1^{+/-}$ or $Rce1^{+/+}$ cells. At the time, it was unclear whether the higher white blood cell counts were truly significant or simply due to a play of chance. In light of the current studies, we suggest that the increased white blood cell counts were in fact due to $Rce1$ deficiency, and we further suggest that $Rce1$ deficiency accelerates the proliferation of both normal and K-RAS$^{G12D}$-expressing hematopoietic cells.

We showed previously that inactivation of $Rce1$ mislocalizes the RAS proteins away from the plasma membrane and reduces the proliferation of skin carcinoma cells, wild-type fibroblasts, and fibroblasts that overexpress a mutationally activated form of RAS (driven by a strong viral promoter). Our current studies reinforced those data: $Rce1$ deficiency reduced the proliferation of fibroblasts in which the latent K-RAS$^{G12D}$ allele was activated (with K-RAS expression driven by the endogenous $Kras2$ promoter). Thus, our studies indicate that $Rce1$ deficiency has distinct effects in fibroblasts and hematopoietic cells.

At this point, the mechanism for the worsened MPD and the increased proliferation of $Rce1$-deficient hematopoietic cells is unknown. The simplest potential explanation would be that RCE1 normally processes an isoprenylated CaaX protein that suppresses cell proliferation and that this protein is dysfunctional in the absence of RCE1-mediated endoproteolytic processing. One potential candidate is RAP1, a RCE1 substrate which is known to be
capable of suppressing RAS signaling\textsuperscript{20}. Regardless of the mechanism, in the future it will be important to determine if \textit{Rce1} deficiency accelerates the development of myeloid leukemia caused by other genetic interventions (e.g. \textit{Nf1} deficiency\textsuperscript{21}). In addition, it will be important to define, with experiments similar to those described here, the \textit{in vivo} importance of the other \textit{CaaX} protein processing enzymes (i.e. FTase, GGTase-I, and ICMT) on the development of MPD and leukemia.

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\section*{References}


Figure legends

Figure 1. Acceleration of K-RAS-induced MPD in the setting of Rce1 deficiency

Groups of Rce1^{fl/fl}K^{LSLM} mice and control Rce1^{fl/+}K^{LSLM} mice were injected at weaning with pI-pC to induce MPD. (A) The concentration of white blood cells was elevated in Rce1^{fl/fl}K^{LSLM} compared with Rce1^{fl/+}K^{LSLM} mice with MPD. Statistically significant changes at each time point are indicated: * P < 0.05; ** P < 0.01; and *** P < 0.001; n = 11–27 per time point [except for the values at 42 days where n = 3 (Rce1^{fl/fl}K^{LSLM}) and n = 5 (Rce1^{fl/+}K^{LSLM})]. (B) Kaplan-Meier curve demonstrating reduced survival of pI-pC-injected Rce1^{fl/fl}K^{LSLM} mice (median survival 40 days; n = 12) compared with Rce1^{fl/+}K^{LSLM} mice (median survival 54 days; n = 5).

Figure 2. Increased release of immature K-RAS^{G12D}-expressing myeloid cells in the setting of Rce1 deficiency

(A) White blood cells were evaluated in blood smears from Rce1^{fl/fl}K^{LSLM} (black bars; n = 6) and Rce1^{fl/+}K^{LSLM} mice (white bars; n = 6) harvested before and after pI-pC injections. Immature cells (ie myeloblasts, promyelocytes, promonocytes, myelocytes, metamyelocytes, band cells, and pelgeroid cells) (A) and lymphocytes (B) are plotted as percent of total white blood cells. Differences between Rce1^{fl/fl}K^{LSLM} and Rce1^{fl/+}K^{LSLM} mice were evident at 3 weeks after pI-pC injection and the proportions did not change over time: thus, data from 3, 5, and 7 weeks were combined. (C) Photographs of blood smears from before and 5 weeks after pI-pC injections (optical magnification, 100×). Upper right panel: arrowhead indicates pelgeroid cell; arrow indicates band cell; the large cell in the center is a myeloblast.
Figure 3. Tissue infiltration of K-RAS<sup>G12D</sup>-expressing myeloid cells

Hematoxylin- and eosin–stained sections of tissues from R<sup>ce1</sup><sup>fl/+</sup> K<sub>LSLM</sub> (left panel) and R<sup>ce1</sup><sup>fl/fl</sup> K<sub>LSLM</sub> mice (right panel) harvested 5 weeks after pI-pC injections. (A), (B) Bone marrow; (C), (D) spleen; (E), (F) liver; (G), (H) lung.

Figure 4. Enhanced colony-forming ability of R<sup>ce1</sup>-deficient K-RAS<sup>G12D</sup>-expressing hematopoietic cells

(A) Spleen weight of R<sup>ce1</sup><sup>fl/fl</sup> K<sub>LSLM</sub> mice (black bars) and R<sup>ce1</sup><sup>fl/+</sup> K<sub>LSLM</sub> mice (white bars) at 3 and 5 weeks after pI-pC injections (n = 6 and 11 for R<sup>ce1</sup><sup>fl/fl</sup> K<sub>LSLM</sub> mice and n = 5 and 8 for R<sup>ce1</sup><sup>fl/+</sup> K<sub>LSLM</sub> mice at 3 and 5 weeks, respectively). Dashed line indicates spleen weight of wild-type mice (4 ± 0.9 mg/g body weight; n = 6). (B), (C) Growth factor–independent colony growth of splenocytes from R<sup>ce1</sup><sup>fl/fl</sup> K<sub>LSLM</sub> (n = 3) and R<sup>ce1</sup><sup>fl/+</sup> K<sub>LSLM</sub> (n = 3) mice; B, colony number; C, colony size. (D) Growth factor-independent colony growth of bone marrow cells (n = 2). (E) Upper panel: Photographs showing R<sup>ce1</sup><sup>Δ/Δ</sup> K<sub>G12DM</sub> and R<sup>ce1</sup><sup>Δ/+</sup> K<sub>G12DM</sub> splenocyte colonies from a typical experiment in B and C. Lower panel: May-Grünwald-Giemsa–stained cytospins of individual colonies. (F) PCR amplification of genomic DNA from individual colonies to detect the R<sup>ce1</sup><sup>fl</sup> and R<sup>ce1</sup><sup>+</sup> alleles (upper panel) and the Kras<sup>2+</sup> and Kras<sup>2G12D</sup> alleles (lower panel). Lane 1, R<sup>ce1</sup>-deficient Kras<sup>2G12D</sup> colony; Lane 2, heterozygous R<sup>ce1</sup>-deficient Kras<sup>2G12D</sup> colony; Lanes 3 and 4, control DNA from mouse tails. The same result was found in 5 additional colonies from spleens of R<sup>ce1</sup><sup>fl/fl</sup> K<sub>LSLM</sub> and R<sup>ce1</sup><sup>fl/+</sup> K<sub>LSLM</sub> mice.

Figure 5. Inactivation of R<sup>ce1</sup> inhibits the growth of K-RAS<sup>G12D</sup>-expressing fibroblasts

(A) Cell proliferation assay of spontaneously immortalized R<sup>ce1</sup><sup>fl/fl</sup> K<sub>LSLM</sub> and R<sup>ce1</sup><sup>fl/+</sup> K<sub>LSLM</sub> embryonic fibroblasts treated with Cre and β-gal-adenoviruses. Data are mean of 2 independent cell lines per genotype. The experiment was repeated with a pair of primary cell lines with similar results. (B) PCR amplification of genomic DNA from the cells in A
demonstrating the activation of the $Kras^{2G12D}$ allele and the inactivation of the $Rce1^{fl}$ allele. 1, β-gal-adenovirus–treated $Rce1^{fl/+}K^{LSL}$ cells; 2, Cre-adenovirus–treated $Rce1^{fl/+}K^{LSL}$ cells; 3, β-gal-adenovirus–treated $Rce1^{fl/fl}K^{LSL}$ cells; 4, Cre-adenovirus–treated $Rce1^{fl/fl}K^{1SL}$ cells.
Figure 1

A

B

White Blood Cells (x10^9/L)

Time (days)

Survival (%)

Time (days)

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Figure 2

A

P < 0.0001

Immature cells (%) - pl-pC + pl-pC

B

P > 0.05

P < 0.001

Lymphocytes (%) - pl-pC + pl-pC

C

- pl-pC + pl-pC

Rce1\textsuperscript{fl/fl}\textsuperscript{K\textsubscript{LSL/M}}

Rce1\textsuperscript{fl+/fl}\textsuperscript{K\textsubscript{LSL/M}}
Figure 3

A: Rce1^{fl/fl} K^{LSLM}
B: Rce1^{fl/fl} K^{LSLM}
C: Rce1^{fl/fl} K^{LSLM}
D: Rce1^{fl/fl} K^{LSLM}
E: Rce1^{fl/fl} K^{LSLM}
F: Rce1^{fl/fl} K^{LSLM}
G: Rce1^{fl/fl} K^{LSLM}
H: Rce1^{fl/fl} K^{LSLM}
Figure 5

A

Number of cells (x 10^3)

Time (days)

B

1 2 3 4

Rce1fl
Rce1^+
Kras2^G12D
Kras2^+
Rce1 deficiency accelerates the development of a K-RAS-induced myeloproliferative disease

Annika M Wahlstrom, Briony A Cutts, Christin Karlsson, Karin M.E. Andersson, Meng Liu, Anna-Karin M. Sjogren, Birgitta Swolin, Stephen G Young and Martin O Bergo