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

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The RAS proteins undergo farnesylation of a carboxyl-terminal cysteine (the “C” of the carboxyl-terminal CaaX motif). After farnesylation, the 3 amino acids downstream from the farnesyl cysteine (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. Therefore, we hypothesized that the 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 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 the inactivation of Rce1 worsens the myeloproliferative disease caused by oncogenic K-RAS. (Blood. 2007;109:763-768)

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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 hematologic malignancies.1,2 For example, activating mutations in RAS are present in as many as 44% of patients with acute myeloid leukemia.3 Hematologic malignancies also occur when RAS signaling is increased, as observed in diseases such as neurofibromatosis (NF1) in which a genetic abnormality develops in a RAS-interacting protein.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 3 amino acids of the protein (the -aaX of the CaaX motif) are released by RAS-converting enzyme 1 (RCE1), and the newly exposed farnesyl cysteine is methyleated by isoprenyl cysteine carboxyl methyltransferase (ICMT). Several RAS isoforms (but not K-RAS4B) are also palmitoylated at nearby cysteine residues.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.6 However, in clinical trials of human solid tumors, FTIs have not met the high expectations, in part because RAS proteins can be isoprenylated by a related isoprenyl transferase, geranylgeranyl transferase type I (GGTase-I), in the presence of an FTI.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 are farnesylated or geranylgeranylated. In addition, this strategy would not be expected to cause significant toxicity in vivo. Indeed, we have inactivated Rce1 in the liver, spleen, and bone marrow of mice and not observed significant adverse effects.8 Ayiagari et al9 showed that Rce1-deficient fetal liver cells are capable of rescuing hematopoiesis in lethally irradiated mice. Several lines of investigation suggest that the inhibition of RCE1 would inhibit RAS-induced oncogenic transformation. First, the inactivation of Rce1 mislocalized the RAS proteins and reduced cell proliferation and the anchorage-independent growth of RAS-transformed fibroblasts in soft agar and nude mice.8,10 Second, in the absence of Rce1, skin carcinoma cells grew slowly and were highly sensitive to the effects of an FTI.8 Third, several potent RCE1 inhibitors have been developed,11,12 and 2 of them reduced the anchorage-independent growth of K-RAS–transformed cells in vitro.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 the inactivation of Rce1 in mice would inhibit the development of K-RAS–induced myeloproliferative disease (MPD) in vivo. To accomplish this, we used Cre recombinase to simultaneously inactivate the expression of Rce1 and activate the expression of oncogenic K-RAS\textsuperscript{G12D} in hematopoietic cells.

Materials and methods

Breeding mice for in vivo experiments

Mice with a conditional Rce1 knockout allele (Rce1	extsuperscript{fl}) have been described.\textsuperscript{8} Mice with a Kras2\textsuperscript{fl} allele have an activating mutation (G12D) in the Kras2 gene and a “floxed” transcriptional terminator sequence upstream in the promoter (LSL;loxP-STOP-loxP). Cre expression results in the removal of the STOP cassette, which turns on the expression of K-RAS\textsuperscript{G12D}. For our experiments, Rce1\textsuperscript{fl} mice with a Kras2\textsuperscript{fl} allele were bred with Rce1\textsuperscript{fl} mice harboring an interferon-inducible Mxi-Cre transgene\textsuperscript{16} to generate Rce1\textsuperscript{fl}Kras2\textsuperscript{fl}/Mxi-Cre mice. In those mice (hereafter designated Rce1\textsuperscript{fl}Kras2\textsuperscript{fl}/Mxi-Cre), Cre expression simultaneously inactivated Rce1 expression and activated the expression of K-RAS\textsuperscript{G12D} in bone marrow cells. These cells are designated Rce1\textsuperscript{fl}Kras2\textsuperscript{fl}/Mxi-Cre (LSL;Kras2\textsuperscript{fl}/Mxi-Cre 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\textsuperscript{fl}Kras2\textsuperscript{fl}/Mxi-Cre mice were littersmate. Animal procedures were approved by the animal research ethics committee in Gothenburg.

Genotyping

Mice were genotyped by polymerase chain reaction (PCR) amplification of genomic DNA obtained by tail biopsy. The Rce1\textsuperscript{fl} allele was detected with forward primer 5′-GCTTTTGGAAGACAGGCCC-3′ and reverse primer 5′-CTACCTCGATGGGTTCAC-3′. This PCR reaction yields a 350-bp fragment from the Rce1\textsuperscript{fl} allele and a 270-bp fragment from the Rce1\textsuperscript{f} allele. The Kras2\textsuperscript{fl} allele was detected with forward primer 5′-GAGCTCCATTCATGTGTGGT-3′ and reverse primer 5′-CTAGAGCCCTGTGGTGTC-3′; the amplified fragment measured 1009 bp. The Kras2\textsuperscript{fl} allele was detected with forward primer 5′-CCTTTCTACAGGCGCAGACTTAGG-3′ and reverse primer 5′-AGCTGACCCATGTGGTTAGTC-3′; the amplified fragment measured 600 bp. The presence of the activated Kras2\textsuperscript{G12D} allele was detected with forward primer 5′-GGAGTTAGGTTGGGATGCTG-3′ and reverse primer 5′-TCCGAATTCACTTAGACTACAGATGTACAGAG-3′; the amplified fragment measured 320 bp.

Quantifying the efficiency of Cre-induced recombination of the Rce1\textsuperscript{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: forward primer 5′-CGAGTTAAATCTTGGGGAGGAGG-3′ and reverse primer 5′-CGGTGCAATAACCTTGGTTAGTC-3′; pggt1b (used as a control) 5′-CATGTGCCCTGCTTTACATA-3′ and reverse primer 5′-CAGTTTACCCATCAGGCCA-3′.

Injection of polyninosic–polycytidylic acid and monitoring

The Mxi-Cre transgene was activated with intraperitoneal injections of 400 μg polyninosic–polycytidylic acid (p-l-pC; Sigma, St Louis, MO) 21 days after birth (at weaning) once every other day, for a total of 4 injections. Blood was drawn from a tail vein before the first p-l-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) and by manual differential counts of May-Grünewald-Giemsa–stained smears by 2 trained observers blinded to genotype (200 white blood cells per slide were evaluated). At 3 and 5 weeks, groups of mice were killed and tissues were harvested.

Histology

Tissues were fixed in 4% PBS-buffered formalin, dehydrated in 70% to 100% ethanol, and cleared in xylene. Tissues were embedded in paraffin and 4- to 5-μm sections were stained with hematoxylin and eosin. Sections were viewed and photographed with a light microscope (AxioCam HR digital camera; Axioplan 2; Carl Zeiss, Oberkochen, Germany) and analyzed with Axiovision AC software version 4.3. Cytoxin preparations of bone marrow cells were stained with May-Grünewald-Giemsa. Cells of the monocytic and granulocytic lineages were identified by staining for nonspecific and specific esterases, respectively, using α-naphthyl butyrate and naphthol AS-D chloroacetate as substrates.

Colony assays

Spleen cells (10\textsuperscript{5}) and bone marrow cells (2 × 10\textsuperscript{4}) harvested 5 weeks after pl-l-pC injections of Rce1\textsuperscript{fl}Kras2\textsuperscript{fl}/Mxi-Cre, Rce1\textsuperscript{fl}Kras2\textsuperscript{fl}/Mxi-Cre transgene mice, and wild-type mice were seeded in duplicate wells in methylcellulose medium (MethoCult M3234; StemCell Technologies, Vancouver, BC, Canada) in the absence of growth factors. Ten days later, numbers and sizes of colonies were scored. Colonies were viewed and photographed with a Leica DMIL light microscope and a Leica DC200 digital camera (Leica, Wetzlar, Germany). Genomic DNA from individual colonies was genotyped by PCR (to detect the activated Kras2\textsuperscript{G12D} allele and the excision of Rce1). Cytoxin preparations of cells in individual colonies were stained with May-Grünewald-Giemsa and analyzed by light microscopy (Axioplan 2).

Fluorescence-activated cell sorting

Peripheral blood mononuclear cells, bone marrow cells, and cells from methylcellulose colonies were incubated with antibodies against cell-surface antigens (CD11b [catalog no. 550993], CD14 [catalog no. 553739], CD45 [catalog no. 557695], and CD117 [catalog no. 553355]; Pharmingen, San Diego, CA) and were analyzed in a FACSaria (BD Biosciences, San Jose, CA). Data were analyzed with CellQuest software (BD Biosciences).

Isolation of mouse embryonic fibroblasts and cell proliferation assays

Mouse embryonic fibroblasts (MEFs) were isolated from Rce1\textsuperscript{fl}Kras2\textsuperscript{fl} and Rce1\textsuperscript{fl}Kras2\textsuperscript{fl} embryos (lacking the Mxi-Cre transgene) at embryonic day 13.5 (E13.5) to E16.5. Experiments were performed on primary (passages 0-3) and on spontaneously immortalized MEFs (passages 15-35). Cells (5 × 10\textsuperscript{4}) were seeded onto 60-mm dishes in the presence of adenovirus encoding Cre or β-gal (10\textsuperscript{9} pfu/mL AdRSVCre and AdRSVlacZ, respectively). Cre-adenovirus treatment of Rce1\textsuperscript{fl}Kras2\textsuperscript{fl} cells produced Rce1\textsuperscript{fl}Kras2\textsuperscript{G12D} cells that expressed endogenous K-RAS\textsuperscript{G12D} but lacked Rce1 expression; Cre-adenovirus treatment of Rce1\textsuperscript{fl}Kras2\textsuperscript{fl} cells produced Rce1\textsuperscript{fl}Kras2\textsuperscript{G12D} cells that expressed endogenous K-RAS\textsuperscript{G12D} and lacked 50% of Rce1 expression. Cells (2 × 10\textsuperscript{4}) 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 (Nucleo-Counter; ChemoMetec, Allerød, Denmark). DNA was extracted from a portion of the cells, and the excision of the Rce1 allele and the appearance of the activated Kras2\textsuperscript{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 MgCl\textsubscript{2}, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% NP-40, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 mM orthovanadate, and the Complete Mini protease inhibitor cocktail [Roche, Basel, Switzerland]). Lysates were homogenized, sonicated, and centrifuged at 20 000 g for 20 minutes, and equal amounts of total protein of the supernatant were size-fractionated on 10% to 20% sodium dodecyl sulfate polyacrylamide gels (Criterion; Bio-Rad, Hercules, CA). The proteins were transferred onto nitrocellulose membranes and incubated...
with antibodies recognizing phosphorylated ERK1/2 (9217), total ERK (9102; Cell Signaling, Danvers, MA), and α-tubulin (clone 12F11; Sigma-Aldrich, St. Louis, MO). Bands were visualized with horseradish peroxidase–conjugated secondary antibody (sc-2314 and sc-2311; Santa Cruz) and the Enhanced Chemiluminescence kit (Amersham, Little Chalfont, Buckinghamshire, United Kingdom).

Statistical analyses

Data are plotted as mean plus or minus 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 t test; survival was assessed by the Mann-Whitney U test.

Results

Inactivation of Rce1 exacerbates K-RAS–induced myeloproliferative disease

To determine whether Rce1 deficiency would inhibit the development of MPD, we monitored pl-pC–injected Rce1fl/flKLSLM mice and control Rce1fl/flKLSLM mice. The pl-pC–injected Rce1fl/flKLSLM mice developed progressive leukocytosis (Figure 1A) with an increased percentage of myeloid cells (Table S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). The pl-pC–injected Rce1fl/flKLSLM mice exhibited an increase in white blood cell counts compared with the Rce1fl/flKLSLM mice; this increase was statistically significant 2 weeks after the pl-pC injections (Figure 1A). At 5 weeks, the mean white blood cell count in Rce1fl/flKLSLM mice was 106 ± 12 × 10^9/L (n = 15) compared with 39 ± 3 × 10^9/L (n = 11) in Rce1fl/flKLSLM mice (P < .001). The increased proliferation of white blood cells in the pl-pC–injected Rce1fl/flKLSLM mice was associated with reduced survival (Figure 1B; P = .035). We concluded that Rce1 deficiency accelerated the development of K-RASG12D–induced MPD and reduced survival.

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

In the pl-pC–treated control Rce1flflKLSLM mice with MPD, the percentage of immature myeloid cells was relatively minor (11% vs 3% before pl-pC injections) (Figure 2; Table S1). In contrast, there was a dramatic increase in the percentage of immature myeloid cells in pl-pC–treated Rce1fl/flKLSLM mice (Figure 2A; Table S1). Overall, 58% of white blood cells in these mice were immature. There was a proportionate reduction in the percentage of lymphocytes in the 2 groups of mice (Figure 2B). Thus, the increase in white blood cell counts in pl-pC–treated Rce1fl/flKLSLM mice relative to control mice with MPD was caused by an accumulation of immature myeloid cells.

To determine whether the enhanced production of immature white blood cells was associated with increased myeloid infiltration into vital tissues, we conducted histologic studies. Five weeks after pl-pC injections, increased cellularity was noted in the bone marrow in Rce1flflKLSLM mice and Rce1fl/flKLSLM mice (Figure 3A-B). Fluorescence-activated cell sorter (FACS) analyses and double-esterase staining of bone marrow demonstrated a greater proportion of CD45+/CD11b+/CD14+ cells expressing specific esterase, 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 Rce1flflKLSLM mice (P = .007) and further increased to 73.5% ± 1.3% in the Rce1fl/flKLSLM mice (P = .01 vs Rce1fl/flKLSLM mice; P = .001 vs wild-type). The proportion of CD45+/CD11b+/CD14+ bone marrow cells increased from 4.5% in wild-type mice to 8.1% in Rce1fl/flKLSLM mice and 9.3% in Rce1fl/flKLSLM mice.

The Rce1flflKLSLM mice exhibited mild to moderate infiltration of leukocytes into the liver and spleen, effacement of splenic architecture with extramedullary hematopoiesis, and adenoma

Figure 1. Acceleration of K-RAS–induced MPD in mice with Rce1 deficiency. Groups of Rce1fl/flKLSLM mice and control Rce1fl/flKLSLM mice were injected at weaning with pl-pC to induce MPD. (A) The concentration of white blood cells was elevated in Rce1fl/flKLSLM mice compared with Rce1fl/flKLSLM mice with MPD. Statistically significant changes at each time point are indicated. *P < .05; **P < .01; and ***P < .001; n = 12 or 11 per time point, except for the values at 42 days (n = 3 [Rce1fl/flKLSLM] and n = 5 [Rce1fl/flKLSLM]). (B) Kaplan-Meier curve demonstrating reduced survival of pl-pC–injected Rce1fl/flKLSLM mice (median survival, 40 days; n = 12) compared with Rce1fl/flKLSLM mice (median survival, 54 days; n = 5).

Figure 2. Increased release of immature K-RASG12D–expressing myeloid cells in the setting of Rce1 deficiency. (A) White blood cells were evaluated in blood smears from Rce1fl/flKLSLM (n = 6) and Rce1fl/flKLSLM mice (n = 6) harvested before and after pl-pC injections. Immature cells (myeloblasts, promyelocytes, myelocytes, metamyelocytes, band cells, and polk cells) (A) and lymphocytes (B) are plotted as percentages of total white blood cells. Differences between Rce1fl/flKLSLM and Rce1fl/flKLSLM mice were evident 3 weeks after pl-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 pl-pC injections (magnification: 100×/1.30 NA oil immersion objective lens). (Top right panel) Arrowhead, polk cell; arrow, band cell; large cell in center, myeloblast.
formation in the lung (Figure 3C,E,G). In the pl-pC-treated \textit{Rce1}^{fl/fl}\textit{KLSLM} mice, severe infiltration of leukocytes into the liver occurred with congestion of central veins, swelling of hepatocytes, and areas of necrosis (Figure 3F). In addition, complete effacement of splenic architecture and adenoma formation with diffuse hyperplasia in the lung—likely contributing factors in the rapid demise of the \textit{Rce1}^{fl/fl}\textit{KLSLM} mice—developed (Figure 3D,H).

To determine the efficiency of Cre-induced recombination in hematopoietic tissues of pl-pC–injected \textit{Rce1}^{fl/fl}\textit{KLSLM}, we performed quantitative PCR of genomic DNA. Efficiency was 95% ± 1.6% in spleen and 90% ± 2.2% in bone marrow (n = 3). Thus, both alleles of \textit{Rce1} were inactivated in most cells in those tissues.

To determine the consequences of inactivating \textit{Rce1} in \textit{K-RASG12D}–expressing tissues on downstream signaling molecules, we performed Western blot analysis. Levels of phosphorylated ERK1/2 in the spleens and livers of pl-pC–injected \textit{Rce1}^{fl/fl}\textit{KLSLM} and \textit{Rce1}^{fl/fl}\textit{KLSLM} mice were increased compared with control mice without MPD (Figure S1). Levels of phosphorylated AKT, p21CIP, and total ERK1/2 were not different.

**Enhanced colony-forming ability of \textit{Rce1}–deficient, \textit{K-RASG12D}–expressing hematopoietic cells**

Spleen weights in \textit{Rce1}^{fl/fl}\textit{KLSLM} and \textit{Rce1}^{fl/fl}\textit{KLSLM} mice were similar 3 and 5 weeks after pl-pC injections (Figure 4A). However, given the more advanced histologic findings in spleens from \textit{Rce1}^{fl/fl}\textit{KLSLM} mice (Figure 3D), we assessed the ability of splenocytes from the mice to form colonies in methylcellulose. \textit{Rce1}^{fl/fl}\textit{KLSLM} splenocytes were capable of forming colonies in the absence of growth factors, though the colonies were small (mean number of colonies, 9 ± 3/10^5 cells; mean size, 0.19 ± 0.02 mm; Figure 4B-C). In the absence of \textit{Rce1}, there was a 6.6-fold increase in colony number and a 3.4-fold increase in colony size (Figure 4D). With bone marrow cells, there was a 2-fold increase in colony number (Figure 4D). Hematopoietic cells from wild-type

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\caption{Enhanced colony-forming ability of \textit{Rce1}–deficient, \textit{K-RASG12D}–expressing hematopoietic cells.}
\end{figure}
mice did not form colonies in the absence of growth factors. Splenocyte colonies from Rce1fl/flKLSL cells and Rce1fl/flKLSL 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 that the Kras2G12D allele was activated (Figure 4F).

**Rce1 deficiency does not enhance the proliferation of K-RASG12D-expressing fibroblasts**

To determine the impact of Rce1 deficiency on the proliferation of other cell types expressing K-RASG12D, we isolated Rce1fl/flKLSL and control Rce1fl/+KLSL MEFs and treated them with a Cre-adenovirus. As expected, Cre-adenovirus treatment of Rce1fl/+KLSL MEFs activated the expression of K-RASG12D and increased cell proliferation (Figure 5A). In experiments with primary and spontaneously immortalized cell lines, the complete inactivation of Rce1 in Rce1fl/+KLSL MEFs attenuated the increased proliferation caused by K-RASG12D. Thus, the inactivation of Rce1 reduced the proliferation of K-RASG12D–expressing MEFs, even though the same genetic intervention increased the proliferation of K-RASG12D–expressing hematopoietic cells.

**Discussion**

We thought the inactivation of Rce1 would inhibit the development of K-RAS–induced MPD in vivo. This hypothesis 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 the infiltration and proliferation of cells in vital tissues. These studies suggested that RCE1 inhibitors would not be useful for the treatment of RAS-induced hematopoietic malignancies. Indeed, such a strategy could be harmful.

Our experiments involved the use of Cre recombinase to simultaneously inactivate Rce1 and activate K-RASG12D. 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 Kras2G12D allele was activated. Moreover, Rce1 was deleted in more than 90% of the cells in spleen and bone marrow, as judged by quantitative PCR.

**Figure 5. Inactivation of Rce1 inhibits the growth of K-RASG12D–expressing fibroblasts.** (A) Cell proliferation assay of spontaneously immortalized Rce1fl/flKLSL and Rce1fl/+KLSL 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 and yielded similar results. (B) PCR amplification of genomic DNA from the cells in panel A demonstrating the activation of the Kras2G12D allele and the inactivation of the Rce1 allele. Lane 1, β-gal-adenovirus-treated Rce1fl/flKLSL cells; lane 2, Cre-adenovirus-treated Rce1fl/+KLSL cells; lane 3, β-gal-adenovirus–treated Rce1fl/flKLSL cells; lane 4, Cre-adenovirus–treated Rce1fl/flKLSL cells.
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Authorship

Contribution: A.M.W. designed and performed the research; B.A.C., K.M.E.A., M.L., and A.K.M.S. performed the research; C.K. analyzed the data; B.S. designed the research and analyzed the data; S.G.Y. designed the research; and M.O.B. designed the research and wrote the paper.

Conflicts-of-interest disclosure: The authors declare no competing financial interests.

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