Erk1 and Erk2 are required for maintenance of hematopoietic stem cells and adult hematopoiesis

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Running title: Erk1 and Erk2 in murine hematopoiesis

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*in vivo*
Abstract

Extracellular signal-regulated kinase 1 (Erk1) and Erk2 play crucial roles in cell survival, proliferation, cell adhesion, migration, and differentiation in many tissues. Here we report that the absence of Erk1 and Erk2 in murine hematopoietic cells leads to bone marrow aplasia, leukopenia, anemia and early lethality. Mice doubly-deficient in Erk1 and Erk2 show rapid attrition of hematopoietic stem cells (HSC) and immature progenitors a cell-autonomous manner. Reconstitution studies show that Erk1 and Erk2 play redundant and kinase-dependent functions in hematopoietic progenitor cells. Moreover, in cells transformed by the oncogenic KRasG12D allele, the presence of either Erk1 or Erk2 with intact kinase activity is sufficient to promote cytokine-independent proliferation.

Introduction

Erk1 and Erk2 (Erk/12) encode serine/threonine kinases belonging to the mitogen-activating protein kinase (MAPK) family, and play critical roles in transmitting signals from a plethora of cytokines and growth factors. ERK1 and ERK2 are >80% identical, and both phosphorylate a myriad of substrates, many of which are cell line- and agonist-specific. Erk1-/- mice are viable and fertile, whereas Erk2-/- mice are embryonic lethal due to placental defects. ERK1/2 activation appears to be required for the survival and differentiation of many types of hematopoietic cells, but most studies of ERK1/2 function in hematopoiesis have employed pharmacological blockade of MEK1 and MEK2, the kinases that activate ERK1/2. Genetic experiments argued that Erk1 is required for T cell development, although this observation is controversial. T cell-specific deletion of Erk2 also leads to defective T lymphocyte maturation, suggesting that in some cellular contexts, ERK1 cannot compensate for ERK2. By contrast, either Erk1 or Erk2 is sufficient for normal B cell development, although
this process is markedly impaired in Erk1/2 doubly-deficient B cells. Together, these observations suggest that ability of Erk1 and Erk2 to compensate for one another might be cell-type specific. To date, the role of Erk1/2 in HSC function remains to be characterized. Here we show that Erk1/2 are required in a cell-autonomous manner for the maintenance of HSC.

Methods

Erk1-/- and Erk2flox/flox mice were crossed to Mx1-Cre (The Jackson Laboratory, Bar Harbor, ME) mice, as indicated. All mice were maintained in accord with guidelines approved by the animal welfare committee of University Health Network.

Results and Discussion

To assess ERK1 and ERK2 function in hematopoiesis, we generated Mx1-Cre;Erk2flox/flox and Mx1-Cre Erk1-/-;Erk2flox/flox mice in C57BL/6J background and induced Cre recombinase expression by administration of polyinosinic-polycytidylic acid (pIpC). Hematopoiesis in untreated or pIpC-treated Erk2flox/flox (hereafter, “Control”) mice was indistinguishable from that in wild-type C57BL/6J animals (data not shown). Most induced Mx1-Cre Erk1-/-; Erk2flox/flox (hereafter, Erk1-/-Erk2Δ/Δ) mice became moribund or died 30–40 days after pIpC injection (Figure 1A), whereas Erk1-/- and induced Mx1-Cre; Erk2flox/flox (hereafter, Erk2Δ/Δ) mice were indistinguishable from Controls. Erk1-/-Erk2Δ/Δ mice were leukopenic, severely anemic, and had markedly reduced numbers of bone marrow (BM) cells (Figures 1B, C, D and S1A). There also was a trend towards decreased numbers of mature myeloid, B cells and erythroid cells in the bone marrow of Erk1-/-Erk2Δ/Δ mice, although this difference did not reach statistical significance (Figure S1B).
Next, we examined the HSC-enriched Lin\(^{-}\)Sca1\(^{+}\)c-Kit\(^{+}\) (LSK) compartment and early progenitors in Control and Erk-deficient BM. Erk1\(^{-}\) and Erk2\(^{Δ/Δ}\) mice showed normal numbers of LSK cells, Common Myeloid Progenitors (CMP), Granulocyte-Macrophage Progenitors (GMP) and Megakaryocyte-Erythrocyte Progenitors (MEP), but all of these progenitors were reduced markedly in Erk1\(^{-}\)Erk2\(^{Δ/Δ}\) BM (Figure 1E). Analysis of a more highly enriched HSC compartment (LSKCD150\(^{-}\)CD48\(^{+}\), SLAM-HSC) also revealed a marked reduction in the frequency and the absolute cell number of these cells in Erk1\(^{-}\)Erk2\(^{Δ/Δ}\) mice (Figure 1F and data not shown), indicating a defect at the earliest stage of adult hematopoiesis. Thus, either Erk1 or Erk2 is sufficient to maintain hematopoiesis, but deletion of both Erk1 and Erk2 results in rapid hematopoietic failure and depletion of HSCs and progenitors.

Next, we investigated the ability of Erk-deficient HSC to respond to cytokines that promote HSC maintenance. SLAM-HSC were isolated from Control and Erk-mutant mice 10 days after pIpC treatment, and placed at one cell per well in a medium that preserves multipotency in vitro\(^{13,14}\). Mutant cells harvested at this time point all showed complete ablation of the appropriate Erk mRNA (Figure S2A, B). After 4 days of culture, >85% of cells from Control, Erk1\(^{-}\), Erk2\(^{Δ/Δ}\) mice were viable and had shown substantial proliferation. By contrast, Erk1\(^{-}\)Erk2\(^{Δ/Δ}\) cells underwent at most one or two cell divisions, and only ~40% remained at the end of the experiment (Figure 2A). These data indicate that compound loss of Erk1 and Erk2 is incompatible with the proliferation and survival of HSC in vitro.

To test whether the requirement for Erk1 and Erk2 in hematopoiesis is cell-autonomous, we transferred uninduced Erk2\(^{lox/lox}\), Erk1\(^{-}\), Mx1-Cre; Erk2\(^{lox/lox}\) and Mx1-Cre; Erk1\(^{-}\); Erk2\(^{lox/lox}\) BM cells, together with wild-type congenic CD45.1-expressing carrier BM, into lethally irradiated CD45.1 animals. Recipients were maintained for 5 weeks to allow establishment of steady-state hematopoiesis. These mice were then injected with pIpC, and the ratio of CD45.1\(^{+}\) versus CD45.2\(^{+}\) cells in the peripheral blood was monitored. All mice showed comparable levels of CD45.2 engraftment before pIpC treatment (Figure 2B). Moreover, the percentage donor
chimerism of the B-cell and myeloid lineages in recipients of Control, Erk1−/−, and Erk2Δ/Δ BM cells did not differ significantly. By contrast, there was a significant reduction in the percentage of donor-derived T cells in recipients of Erk1−/− or Erk2Δ/Δ BM cells, with Erk2Δ/Δ BM cells recipients showing a more pronounced defect. Finally, mice that had received Mx1-Cre Erk1−/−; Erk2Δ/Δ/Δ BM cells showed profound attrition of donor-derived T, B and myeloid cells following plpC treatment, indicating that the defect in HSC maintenance in the absence of ERK1 and ERK2 is cell-autonomous.

To further assess the effects of Erk deficiency on the functional competence of HSC, we performed competitive reconstitution studies. In these experiments, Control or Erk-deficient BM cells were mixed with CD45.1+ BM cells at 1:1 ratio before they were injected to lethally-irradiated CD45.1+ recipients. Beginning eight weeks after transplantation, CD45.2-derived cells in the peripheral blood of recipient mice were quantified. Recipients of Erk1−/− and Erk2Δ/Δ BM cells showed a reduced proportion of donor-derived T and B cells (Figure 2C). However, the frequencies of donor-derived myeloid cells in recipients of Control, Erk1−/− and Erk2Δ/Δ BM cells were similar. By contrast, animals that received Erk1−/−Erk2Δ/Δ BM cells had almost no contribution of donor-derived cells from any lineage in their peripheral blood. Thus, single Erk knockout HSC and myeloid progenitors are not disadvantaged under competitive conditions, but, deletion of both Erk isoforms leads to gross attrition of all hematopoietic lineages.

Although Erk2 is known to play a crucial role in T cell development and activation9,11, the role of Erk1 in T lineage cells remains controversial4,5,9,11. Importantly, the development and maintenance of Erk1-deficient T cells in an Erk1-sufficient background has never been examined. Our data suggest that both Erk1 and Erk2 are necessary for normal T cell development/maintenance in vivo. Furthermore, our data indicate that the development and maintenance of Erk1- and Erk2-deficient B cells are diminished in the presence of wild-type B cells, indicating that B lymphocytes lacking a single ERK isoform have a subtle functional impairment not revealed in the non-competitive setting.
Isoform-specific functions of *Erk1* and *Erk2* remain controversial\textsuperscript{2,3}. Moreover, ERK1 and ERK2 are known to have kinase-independent functions\textsuperscript{15}. To ask whether *Erk1* and *Erk2* have unique functions in hematopoietic cells and whether catalytic activity is required for hematopoietic progenitor function, we constructed retroviral vectors co-expressing a GFP-Cre fusion protein and either the hygromycin resistance gene (*hygro*), *Erk1*, *Erk2*, *Erk1\textsuperscript{K72R}* or *Erk2\textsuperscript{K52R}*.*Erk1\textsuperscript{K72R}* and *Erk2\textsuperscript{K52R}* encode kinase-defective mutants of ERK1 and ERK2\textsuperscript{16}.

Lineage-negative (lin\textsuperscript{-}) BM cells from *Erk1\textsuperscript{-/-}Erk2\textsuperscript{flox/flox}* mice were infected with these viruses, and GFP\textsuperscript{+} cells were purified by FACS and plated in liquid medium containing IL-3, IL-6 and SCF (Figure 2D). In infected cells, GFP-Cre excises the endogenous *Erk2* alleles, generating *Erk1\textsuperscript{-/-}Erk2null* cells, while concomitantly, different *Erk* isoforms are expressed under the control of the same viral promoter. We observed nearly complete loss of cell proliferation in *Erk1\textsuperscript{-/-}Erk2null* cells co-expressing GFP-Cre and *hygro*. Co-expression of wild type *Erk1* or *Erk2* with GFP-Cre restored cell proliferation to a similar extent. In sharp contrast, cells co-expressing kinase-defective *Erk1* or *Erk2* showed minimal proliferation. Thus *Erk1* and *Erk2* have redundant, kinase-dependent functions in hematopoietic progenitors.

Pharmacological blockade of MEK attenuates *KRasG12D*-evoked myeloproliferative disease in mice\textsuperscript{17}. However, the specific role(s) of *Erk1* and *Erk2* in *KRasG12D*-transformed hematopoietic cells remained be to be addressed. Notably, *Erk2* has allele-specific effects on Ras-induced epithelial-to-mesenchymal transformation (EMT)\textsuperscript{18}, raising the possibility that a single ERK isoform might be important for *KRAS*-evoked transformation of hematopoietic cells. To test this possibility, we infected lin\textsuperscript{-} BM cells from *Erk1\textsuperscript{-/-}Erk2\textsuperscript{flox/flox}LSL-KRasG12D* mice with the retroviral vectors described above. In infected cells, *Cre* expression promotes simultaneous deletion of *Erk2* and expression of *KRasG12D* under the control of its endogenous promoter. GFP\textsuperscript{+} cells were purified by FACS and plated in media containing no cytokines to assess *KRasG12D*-evoked cytokine-independent proliferation. Comparable cell proliferation was observed in cells co-expressing either wild-type *Erk1* or *Erk2* (Figure 2E). By contrast, cells
expressing catalytically defective ERK1 or ERK2 showed a complete loss of \( KRas^{G12D} \)-evoked cytokine-independent proliferation. Thus, myeloid cell transformation by oncogenic KRAS requires catalytically active ERK1 or ERK2.

In summary, we found that deletion of both Erk genes results in the rapid loss of HSC and progenitors, leading to leukopenia, anemia, and early lethality in mice. Similar data were reported recently by Staser et al\(^\text{19} \). Together, these results are consistent with previous findings reported by us and others that hematopoietic-specific loss of \( Pttn11 \) (encoding SHP2), a positive regulator of RAS/ERK signaling, also leads to attrition of HSC and progenitors\(^\text{14,20} \).

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**Authorship**

Contribution: G.C. designed research, performed experiments, analyzed data, and wrote the paper. S.G. performed experiments and analyzed data. B.G.N. designed research, analyzed data, and wrote the paper.

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References

Figure 1. Deletion of Erk1 and Erk2 leads to fatal bone marrow failure and loss of HSC. (A) Kaplan-Meier survival analysis of a cohort of Erk1<sup>−/−</sup> (n=23), Mx1-Cre;Erk<sup>flox/flox</sup> (n=21) and Mx1-Cre Erk1<sup>−/−</sup>; Erk2<sup>flox/flox</sup> (n=22) and littermate control (n=18) mice following plpC treatment. Erk1<sup>−/−</sup>-Erk2<sup>Δ/Δ</sup> mice have a median lifespan of 30 days; control mice remain healthy for >120 days. * p<0.05, Logrank test. (B) Hematoxylin and eosin-stained sections from humeri of control, Erk1<sup>−/−</sup>, Erk2<sup>Δ/Δ</sup> and Erk1<sup>−/−</sup>-Erk2<sup>Δ/Δ</sup> animals 30 days after receiving their last dose of plpC. (C, D) White blood cell count (WBC) and hematocrit (HCT) of control, Erk1<sup>−/−</sup>, Erk2<sup>Δ/Δ</sup> and Erk1<sup>−/−</sup>-Erk2<sup>Δ/Δ</sup> mice (* p<0.05, ANOVA). (E) Absolute number (mean±SEM, * p<0.05, ANOVA) of LSK, CMP (Lin−Sca1−cKit−CD34−FcγR<sup>lo</sup>), GMP (Lin−Sca1−cKit−CD34−FcγR<sup>hi</sup>), MEP (Lin−Sca1−cKit−CD34−FcγR<sup>−/lo</sup>) of control (n=6), Erk1<sup>−/−</sup> (n=5), Erk2<sup>Δ/Δ</sup> (n=5) and Erk1<sup>−/−</sup>-Erk2<sup>Δ/Δ</sup> mice (n=6). * p<0.05, ANOVA. (F) Absolute number (mean±SEM, * p<0.05, Student’s t test) of SLAM-HSC from control (n=6), Erk1<sup>−/−</sup> (n=5), Erk2<sup>Δ/Δ</sup> (n=5) and Erk1<sup>−/−</sup>-Erk2<sup>Δ/Δ</sup> mice (n=6). * p<0.05, ANOVA.

Figure 2. Requirement of Erk1 and Erk2 in HSC and immature progenitors. (A) Single SLAM-HSC from of control, Erk1<sup>−/−</sup>, Erk2<sup>Δ/Δ</sup> and Erk1<sup>−/−</sup>-Erk2<sup>Δ/Δ</sup> mice were sorted into 60-well plates (1 cell/well) and cultured in media containing Scf, Tpo, Flt3l and IL-11. Proliferation of each clone was evaluated microscopically over four days. Representative data from one of four experiments with similar results are shown. The average percentages (±SEM) of surviving clones from the four experiments are shown at the top of each panel. * p<0.05, ANOVA. (B) Lethally-irradiated CD45.1<sup>+</sup> recipients (6 mice/group) were transplanted with 10<sup>6</sup> bone marrow cells from control, Erk1<sup>−/−</sup>, Erk2<sup>Δ/Δ</sup> and Erk1<sup>−/−</sup>-Erk2<sup>Δ/Δ</sup> mice, along with 10<sup>5</sup> wild type CD45.1<sup>+</sup> cells. After 5 weeks of engraftment, chimeric mice were treated with five doses of plpC, and the percentage of donor-derived peripheral blood cells (Total), CD3<sup>+</sup> (T cells), B220<sup>+</sup> (B cells), and Gr1<sup>+</sup> (Myeloid cells) were determined flow cytometry. * p<0.05, ANOVA. (C) Control, Erk1<sup>−/−</sup>, Erk2<sup>−/−</sup> and Mx1-Cre Erk1<sup>−/−</sup>-Erk2<sup>flox/flox</sup> mice were treated with plpC for 14 days before their BM
cells (5 x 10^5) were harvested and mixed with equal numbers of wild type CD45.1+ cells and intra-femorally injected into lethally-irradiated CD45.1+ recipients (5 mice/group). After 8 weeks of engraftment, the percentage of donor-derived peripheral blood cells (Total), CD3+ (T cells), B220+ (B cells), and Gr1+ (Myeloid cells) were determined flow cytometry. *p<0.05, ANOVA

(D) Primary lin^- BM cells from Erk1^-; Erk2^flox/flox mice were transduced with the indicated retroviruses, and FACS-purified GFP+ cells were cultured in IMDM medium containing IL-3, IL6, and Scf. MIG-Cre vectors co-express the hygromycin resistance gene (hygro), Erk1, Erk2, Erk1^KR, and Erk2^KR. Relative cell proliferation in each experiment is normalized to that of Erk2. Results are shown as mean±SEM of four independent experiments. *p<0.05, ANOVA. (E) Lin^- BM cells from Erk1^-; Erk2^flox/flox;LSL-Kras^{G12D} mice were transduced with retroviruses as described in (D), and GFP+ cells were cultured in IMDM medium containing no cytokines. Cells were harvested and proliferation was determined. Cell proliferation in each experiment is normalized to that of Erk2 and results are shown as mean±SEM of four independent experiments. *p<0.05, ANOVA.
**Figure 2**

A. Control, Erk1−/−, Erk2−/−, Erk1−/−Erk2−/−

B. Total, T Cells, B cells, Myeloid cells

C. Total, T Cells, B cells, Myeloid cells

D. Percentage of cells per well

E. Percentage of cells over time

Legend:
- **Erk2flox/flox**
- **Erk1−/−**
- **Mx1-Cre; Erk2flox/flox**
- **Mx1-Cre; ErkF1−/−; Erk2flox/flox**
- **Control**
- **Erk1−/− Erk2−/−**

Statistical significance:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
- **** p < 0.0001
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