Expression of oncogenic K-ras from its endogenous promoter leads to a partial block of erythroid differentiation and hyperactivation of cytokine-dependent signaling pathways

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**Running Title:** endogenous oncogenic K-ras in erythroid differentiation

**Author roles:**
- Jing Zhang: experimental design & execution, writing manuscript
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- David A. Tuveson: development of knock-in mice
- Rudolf Jaenisch: development of knock-in mice
- Tyler E. Jacks: development of knock-in mice
- Harvey F. Lodish: experimental design, writing manuscript

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Abstract

When overexpressed in primary erythroid progenitors, oncogenic Ras leads to the constitutive activation of its downstream signaling pathways, severe block of terminal erythroid differentiation, and cytokine-independent growth of primary erythroid progenitors. However, whether high-level expression of oncogenic Ras is required for these phenotypes is unknown. To address this issue, we expressed oncogenic K-ras (K-ras_{G12D}) from its endogenous promoter using a tetracycline-inducible system. We show that endogenous K-ras_{G12D} leads to a partial block of terminal erythroid differentiation in vivo. In contrast to results obtained when oncogenic Ras was overexpressed from retroviral vectors, endogenous levels of K-ras_{G12D} fail to constitutively activate but rather hyperactivate cytokine-dependent signaling pathways, including Stat5, Akt, and p44/42 MAPK, in primary erythroid progenitors. This explains previous observations that hematopoietic progenitors expressing endogenous K-ras_{G12D} display hypersensitivity to cytokine stimulation in various colony assays. Our results support efforts to modulate Ras signaling for treating hematopoietic malignancies.
Introduction

Deregulated Ras signaling frequently occurs in human patients with acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), and myeloproliferative disorders (MPD) (reviewed in 1). Dysregulated Ras signaling is mainly achieved through two mechanisms. First, Ras signaling is aberrantly activated by constitutive activation of upstream tyrosine kinases (e.g. tel-PDGFRβ and BCR-ABL) and tyrosine phosphatases (e.g. PTPN11) or inactivation of NF1, a Ras GTPase activating protein (reviewed in 2). Second, elevated Ras signaling results from oncogenic mutations in the N- and K-ras genes.

In human patients with various hematopoietic malignancies, the red cell lineage is often affected, characterized by different defects in erythroid differentiation 3. We have been using mouse fetal liver erythroid progenitors as a model system to study the role of oncogenic Ras signaling in erythroid differentiation 4,5. In this system, we can monitor erythroid differentiation step-by-step in vivo and in culture based on the expression of erythroid-specific TER119 and non-erythroid specific CD71 (transferrin receptor) surface proteins. We can purify large amounts of erythroid progenitors and early erythroblasts from mouse fetal livers with ~75-85% purity. We can culture purified erythroid progenitors in vitro such that they undergo normal terminal proliferation and differentiation.

Using these tools, we previously showed that overexpression of oncogenic Ras leads to the constitutive activation of its downstream signaling pathways, including p44/42 MAPK, Akt, and Rlf, a severe block of terminal erythroid differentiation, and cytokine-independent growth of primary erythroid progenitors. However, these results might not mimic the conditions in human cancer patients carrying oncogenic Ras mutations in their endogenous loci. To better model human cancers, we used a tetracycline-inducible system to induce K-rasG12D expression from its endogenous locus. Here we show that expression of endogenous K-rasG12D leads to a partial block of terminal erythroid differentiation and hyperactivation of cytokine-dependent signaling pathways.

Materials and Methods

Mice. All mouse lines were maintained in mixed 129sv/Jae and C57BL/6 genetic backgrounds. The embryos were generated from crossing R26-M2rtTA/+;LSL K-rasG12D/+ mice with LC-1/LC-1;R26R/R26R mice 6-9. The pregnant mice were fed with water containing 0.2% doxycycline and 1% sucrose at E13.5. The embryos were harvested 42-48 hours later. Genotyping of the adult mice and embryos was performed as described 6-9.
**Detection of β-galactosidase-neo (βgeo) activity.** TER119-negative cells were purified from individual embryos as previously described. βgeo activity in the purified cells was detected with a fluorogenic substrate fluorescein di β-D-galactopyranoside (FDG) (Invitrogen Molecular Probes, Carlsbad, California) as described.

**Immunostaining and flow cytometric analysis.** Fetal liver cells (FLC) were isolated from E15.5 embryos and were simultaneously stained for CD71 and TER119 as previously described. Flow cytometry was carried out on a Becton Dickinson FACSCalibur machine (BD Biosciences, Franklin Lakes, NJ).

**Western blot Analysis.** TER119-negative FLCs were purified from individual embryos and purified cells were divided into four aliquots. Cells were starved in IMDM containing 0.5%BSA for 30 minutes at 37°C and then stimulated with or without 0.3-3 U/ml Epo for 10 minutes at 37°C. Western blot analysis was performed essentially as described.

**Results and Discussion**

In preliminary studies we used different cre lines to express oncogenic K-ras from its endogenous promotor at different embryonic stages. Because germline expression of endogenous K-rasG12D results in early embryonic lethality, we first used the Mox2 cre line, in which cre expression starts at E5 and is restricted to epiblasts. The late onset and the exclusion of the placenta from cre expression has been reported to rescue the early embryonic lethality seen in Rb (retinoblastoma) germline knockouts. However, embryos carrying both the LSL K-rasG12D and the cre alleles died around E11.5 in mixed 129sv/Jae and C57BL/6 genetic backgrounds (data not shown). Since the first wave of definitive erythropoiesis in mouse fetal liver occurs at E12, the early lethality of these embryos prevented us from studying oncogenic K-ras signaling in primary erythroid progenitors.

To overcome this problem, we used a tetracycline-inducible system (Figure 1A). In this system, expression of a modified tetracycline-controlled transactivator (M2rtTA) is under the control of the Rosa26 promoter (R26-M2rtTA line). In the presence of doxycycline (Dox), M2rtTA binds to tet operators (TetO) and presumably results in cre expression throughout the embryos. To test the recombination efficiency of this inducible system in fetal liver erythroid progenitors (TER119-negative cells), we used a conditional β-galactosidase-neo (βgeo) reporter line (R26R line). The expression of βgeo was detected with its fluorogenic substrate FDG (fluorescein di-β-galactopyranoside). Mouse embryos carrying all three alleles (R26-M2rtTA, TetO-cre, and R26R) were generated. In the absence of Dox, the percent of FDG-positive cells in TER119-negative population was, as expected, close to zero (Figure 1B). In contrast, >90% of TER119-negative cells (predominantly primitive progenitor cells including mature BFU-Es and CFU-Es) became FDG-positive upon Dox induction for as short as one day. Moreover, the floxed K-ras allele was undetectable in the embryos by PCR after Dox treatment (data not shown). Thus, this inducible system can be used to efficiently remove a floxed stop cassette and induce gene expression in early erythroid cells.

Induction of oncogenic K-ras is achieved by feeding the pregnant mice with water containing Dox. We determined the optimal induction scheme to be starting induction on E13.5 and harvesting FLCs two days later (on E15.5).
induction, FLCs were isolated from individual embryos and analyzed by FACS. The percentage of TER119-negative cells (erythroid progenitors and early erythroid blasts) in embryos expressing oncogenic K-ras significantly increased compared to that in wild-type embryos (22.0% and 10.2%, respectively; p<10^-6) (Figures 2A and 2B), suggesting a partial block of terminal erythroid differentiation in vivo. Thus, these embryos showed anemia rather than erythrocytosis due to inefficient erythroid differentiation. This result is consistent with the inefficient erythropoiesis phenotype observed in adult mice expressing endogenous K-ras^G12D^ \textsuperscript{16}.

We further purified TER119-negative cells from individual embryos and stimulated them with or without Epo (Figure 2C). In the absence of Epo stimulation, there was no constitutive activation of downstream signaling pathways in cells expressing oncogenic K-ras. When stimulated with increasing concentrations of Epo, wild-type cells showed increasing activation of the three downstream signaling pathways tested, Stat5, Akt, and p44/42 MAPK, and maximum activation was achieved at 3 U/ml of Epo. In contrast, in cells expressing oncogenic K-ras signaling pathways were hyperactivated at low concentrations of Epo and maximum activation was observed at 1 U/ml of Epo. These data indicate that oncogenic K-ras expressed from its endogenous locus does not lead to constitutive activation of downstream signaling pathways but does hyperactivate them upon Epo stimulation.

The hyperactivation of cytokine-dependent signaling pathways by endogenous K-ras^G12D^ is in sharp contrast to the constitutive activation of Ras downstream signaling pathways seen with overexpressed oncogenic Ras \textsuperscript{5}. Consequently, expression of endogenous K-ras^G12D^ only leads to a mild block of terminal erythroid differentiation and mild hyperproliferation of primary erythroid progenitors in our in vitro culture system (data not shown). Importantly, endogenously expressed K-ras^G12D^ does not support cytokine-independent growth of these cells (data not shown) in contrast to overexpression of oncogenic Ras \textsuperscript{4}.

The hyperactivation of cytokine-dependent signaling pathways by endogenous K-ras^G12D^ is consistent with previous observations that hematopoietic progenitors expressing endogenous K-ras^G12D^ are hypersensitive to cytokine stimulation in various colony assays \textsuperscript{16-18}. To our knowledge, this is the first time that endogenous K-ras^G12D^ is shown to hyperactivate cytokine-dependent signaling pathways in primary hematopoietic progenitors.

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References

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Figure Legends

Figure 1. Expression of oncogenic K-ras can be efficiently induced from its endogenous promoter by a Tet-on system. (A) Schematic representation of the tetracycline-inducible system, which involves in breeding three lines together. The first line is the M2rtTA transactivator driven from the endogenous Rosa26 promoter (R26-M2rtTA). The second line contains two alleles, cre recombinase driven by the Tet operator (LC-1) and a floxed β-galactosidase-neo (βgeo) reporter cassette at the endogenous Rosa26 locus (R26R). The third line is the conditional oncogenic K-ras at its endogenous locus. (B) TER119-negative cells were purified from individual embryos that carried all the alleles and were induced with or without doxycycline (Dox) for 42-48 hours. βgeo activity was detected by a fluorogenic substrate fluorescein di β-D-galactopyranoside (FDG).

Figure 2. Expression of oncogenic K-ras from its endogenous promoter leads to a partial block of erythroid differentiation and hyperactivation of EPO-dependent signaling pathways. At E15.5, fetal liver cells were isolated from individual embryos that had been induced with doxycycline for the previous 42-48 hours. Cells were simultaneously stained for CD71 and TER119 and erythroid differentiation was analyzed (A). The percentages of R1+R2 cells (predominantly primitive progenitor cells, including mature BFU-Es and CFU-Es) are shown in brackets on individual graphs (A) and further quantified (B). (C) TER119-negative cells were purified from individual E15.5 embryos, deprived of serum and growth factors for 30 minutes, then stimulated with various concentrations of Epo for 10 minutes. Phosphorylated and total levels of Stat5, p44/42 ERK, and Akt proteins were measured by Western blotting (see Materials and Methods).
Figure 2.

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