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Retinoic acid, hypoxia, and GATA factors cooperatively control the onset of fetal liver erythropoietin expression and erythropoietic differentiation

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Abstract

The cytokine erythropoietin (Epo) is an essential factor promoting the survival, proliferation, and differentiation of erythroid progenitor cells. Epo expression and the initial phase of definitive erythropoietic differentiation in the fetal liver (E9–E12) are compromised in mouse embryos lacking the retinoic acid receptor RXRα. Our previous work demonstrated that the Epo gene is a direct target of retinoic acid action, via a retinoic acid receptor binding site in the Epo gene enhancer. However, Epo expression and erythropoietic differentiation become normalized in RXRα mutants from E12. In this study, we have investigated the molecular mechanisms underlying the transition in Epo gene regulation from RXRα-dependence to RXRα-independence. We find that three independent regulatory components are required for high level Epo expression in the early fetal liver: ligand-activated retinoic acid receptors, the hypoxia-regulated factor HIF1, and GATA factors. By E11.5, the fetal liver is no longer hypoxic, and retinoic acid signaling is no longer active; Epo expression from E11.5 onward is enhancer-independent, and is driven instead by basal promoter elements that provide a sufficient level of expression to support further erythropoietic differentiation.

Keywords: Erythropoiesis; Erythropoietin; Fetal liver; Retinoic acid; Hypoxia; HNF4

Introduction

The generation of red blood cells (erythropoiesis) is an essential process in vertebrate embryogenesis to progress from diffusion-limited growth to circulatory system-mediated growth. Erythropoiesis involves the commitment of a pluriopotent hematopoietic stem cell to the erythroid lineage, followed by progression through erythroid progenitor and several erythroblast stages, and ultimately culminating in the terminally differentiated erythrocyte. In mouse embryos, erythropoiesis begins in the yolk sac around embryonic day 7.5 (E7.5), concurrent with the initiation of heart contractions. This phase is called primitive erythropoiesis, and concludes around E11.5 with the establishment of definitive erythropoiesis in the embryo proper, although primitive (yolk sac-derived) erythrocytes persist for another 2 or 3 days. Definitive erythropoiesis initiates in the fetal liver around E9.5 (Houssaint, 1981; Palis et al., 1999), concurrent with the formation of the liver from the hepatic diverticulum of the caudal foregut, via colonization by hematopoietic stem cells. In late gestation, definitive hematopoiesis migrates to the bone marrow where it persists throughout postnatal life.

The cytokine erythropoietin (Epo), which is primarily produced by fetal liver hepatocytes and by interstitial cells

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of the adult kidney, plays a critical role in definitive erythropoiesis. Epo contributes to the survival and proliferation of erythropoietic progenitor cells in the fetal liver and is further required for these cells to reach the terminal steps of definitive erythropoiesis. Thus, erythroid progenitor populations are reduced in number and no fetal liver-derived erythroblasts or red blood cells form in Epo$^{-/-}$ embryos (Wu et al., 1995). Epo is not required for primitive erythropoiesis, in that mouse embryos lacking Epo support (albeit at a reduced level) yolk sac production of red blood cells. Epo-deficient embryos die of anemia at E13.5, when the yolk sac-derived red blood cells that sustained development from E7.5–E12.5 die out and are not replaced through definitive erythropoiesis. These embryos show a characteristic small and pale fetal liver at E12.5 and E13.5, a consequence of the absence of terminal erythropoietic differentiation in the liver.

One of the known physiological regulators of Epo expression is hypoxia (Bunn et al., 1998). A reduced oxygen level results in the stabilization of the transcription factor HIF1α (hypoxia-inducible factor 1α) (Semenza, 2001), which heterodimerizes with the hypoxia-independent subunit HIF1β (also known as ARNT) and binds to a defined HIF1 response element in the 3′ enhancer of the Epo gene (Pugh et al., 1991; Semenza et al., 1991) to upregulate expression. In the fetal liver, Epo is expressed primarily by hepatocytes (Koury et al., 1991), and hypoxia-regulated expression is conserved in hepatocellular carcinoma cell lines such as Hep3B and HepG2 (Goldberg et al., 1987). Adjacent to the HIF1 binding site in the mouse Epo 3′ enhancer is the sequence TGACCTctTGACCC, which is known as a DR2 element because of the direct repeat of the hexameric sequence TGACC(C/T) spaced by two nucleotides. The Epo enhancer DR2 element is required for full hypoxic regulation of the Epo gene, although it is not itself responsible for sensing hypoxia (Blanchard et al., 1992). DR2 elements are known binding sites for some members of the nuclear receptor family, and the orphan nuclear receptor HNF4 (hepatocyte nuclear factor-4) has been considered to be the primary factor which is responsible for Epo gene regulation through the DR2 element (Galson et al., 1995). HNF4 is expressed in the fetal liver and postnatal kidney, both sites of Epo gene expression, and is expressed in Hep3B and HepG2 cells as well. Furthermore, forced expression of HNF4 in transfected HeLa cells (which do not normally express HNF4) supports hypoxic inducibility of an Epo reporter construct. HNF4 appears to function synergistically with HIF1 on the Epo enhancer by direct protein–protein interaction and through the recruitment of transcriptional coactivators (Bunn et al., 1998).

We have studied the biological function of the retinoic acid receptor, which is comprised of a heterodimer of RAR and RXR, both members of the nuclear receptor family (Evans, 1988). Mouse embryos lacking the RXRα gene show a completely penetrant fetal liver phenotype at E11.5–E12.5 that is comparable to the fetal liver phenotype of Epo-deficient embryos, including a pale appearance, extensive death of erythroid progenitors, and failure of erythropoietic differentiation (Makita et al., 2001; Sucov et al., 1994). We demonstrated previously (Makita et al., 2001) that the Epo enhancer DR2 element is a direct target of retinoic acid receptor action: Epo mRNA levels are substantially reduced (at least 10-fold) in the fetal liver of RXRα$^{-/-}$ embryos at E10.25, and are retinoic acid inducible in wild type embryonic liver tissue or in primary hepatocyte obtained from wild type embryos at E10.25; furthermore, the Epo enhancer DR2 element is a binding site for RXR–RAR heterodimers, and in transient transfection assays this element confers retinoic acid responsiveness to a reporter gene, and is required for RA responsiveness of an Epo promoter/enhancer reporter gene. Finally, while heterozygotes for RXRα or for Epo are phenotypically normal, RXRα/Epo double heterozygotes are compromised in fetal liver erythroid differentiation, demonstrating genetic interaction. Thus, retinoic acid and RA receptors directly regulate Epo gene expression at the transcriptional level in the fetal liver, and the phenotype of RXRα mutants represents a deficiency of Epo expression.

However, the erythropoietic deficiency of RXRα$^{-/-}$ embryos is transient. Epo gene expression in the fetal liver of mutant embryos becomes normalized relative to control littermates at E12.5, and the fetal liver cellular phenotype of mutant embryos becomes normalized by E13.5 with recovery of erythroid progenitor cell proliferation and differentiation. Thus, while the early phase (E9.5–11.5) of Epo expression is clearly under retinoic acid control, by E12.5 the Epo gene is regulated by RXRα-independent mechanisms. We previously suggested (Makita et al., 2001) that HNF4 might supplant RAR/RXR function in fetal liver beginning around E12.5. As noted above, HNF4 and RAR/RXR bind to the same DR2 element in the Epo gene enhancer, and transient transfection assays indicated that HNF4 could compete with RAR/RXR for binding to this sequence.

In this study, we have identified the transcriptional components that control Epo gene expression and that are responsible for the transition from retinoic acid and RXRα-dependency in the early E9.5–11.5 phase of definitive erythropoiesis to RXRα-independent control thereafter. We show that the Epo gene is regulated by three synergizing factors: RXR/RAR binding to the enhancer DR2 element, HIF1 binding to the adjacent hypoxia response element of the enhancer, and GATA factors which bind to a proximal promoter element. The early fetal liver contains high levels of RARs, RXRα, HIF1, and GATA factors, as well as the enzymatic components needed for RA synthesis, and is hypoxic as well. Although these conditions change by E12, we find that HNF4 does not supplant RAR/RXR to mediate Epo expression. Rather, Epo expression becomes enhancer-