Volume 3, Issue 2, April 2012

Article

Erythropoietin (Epo), Erythropoietin Receptor (EpoR) and Red Cell Development-Deciphering Molecular Connections

*Ramneek Singh, Zarina Begum & Sukhjeet Kaur

Department of Biotechnology, SUS College of Engineering and Biotechnology, Tangori-140306, Punjab, India.

1. Erythropoietin (Epo), Erythropoietin Receptor (EpoR) and Red Cell Development-Deciphering Molecular Connections

Epo and the EpoR are essential for proliferation and differentiation of committed erythroid progenitors, as is the cytosolic protein-tyrosine kinase JAK-2. JAK-2 binds to the EpoR cytosolic domain in the endoplasmic reticulum and facilitates its folding to promote cell surface expression. EpoRs exist on the cell surface as inactive dimers; Epo binding changes their conformation, leading to JAK-2 transphosphorylation and activation. JAK-2 activates many signaling proteins including PI-3' kinase, the transcription factor Stat5, and the Ras pathway. These pathways interact to prevent apoptosis of committed erythroid progenitors allowing them to undergo a predetermined program of terminal proliferation and erythroid differentiation. Stat5 directly activates transcription of the anti-apoptotic protein bcl-xL. Stat5-/-mice exhibits fetal anemia and increased apoptosis of erythroid progenitors caused by reducing bcl-xL levels. Adult $Stat5^{-/-}$ mice are anemic and deficient in generating high erythropoietic rates in response to stress. Thus, Stat5 controls one rate-determining step regulating early erythroblast survival. Activation of the PI-3' kinase pathway leads to activation of the Akt kinase and then phosphorylation and inhibition of FOXO3a, a member of the Forkhead transcription factor family. FOXO3a, in turn, activates transcription of Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). It means that inhibition of TRAIL production by Epo addition partially rescues cells from apoptosis, demonstrating the importance of this pathway in red cell formation.

Activation of dimeric EpoR by Epo binding is achieved by reorienting the EpoR transmembrane and connected cytosolic domains and that certain disulfide-bonded dimers represent the activated dimeric conformation of the EpoR, constitutively activating downstream signaling. There is a need to determine the structure of peptides corresponding to these dimeric active helixes, as this would shed light on the structure of the Epo- activated receptor transmembrane domain.

What is the mechanism of Epo degradation, in erythroid cells expressing the EpoR? Why certain commercially important mutant Epo's with extra carbohydrate chains have a longer biological lifetime? Epo degradation requires expression of the EpoR. A fraction of the Epo bound to surface receptors is internalized by endocytosis and degraded in lysosomes. Most, however, either dissociates from the surface receptor into the medium or is internalized but resecreted. Long-lived mutant Epo binds slower and dissociates more rapidly from surface Epo receptors, but otherwise, the kinetics of internalization, resecretion, and degradation are indistinguishable from normal Epo. The altered receptor-binding kinetics can explain its longer half-life *in vivo*. There is a need to examine the fate of Epo and its long-lived variants with altered numbers of Epo receptors in both hematopoietic and non-hematopoietic cells. This will discern the role of surface EpoRs in normal Epo turnover.

Expression of a dominant-negative H-ras in CFU-E progenitors, or addition of an inhibitor of the MAP kinase pathway, did not affect erythroid differentiation, indicating that activation of the Ras- MAPK pathway by Epo is not essential for erythroid development. K-$ras^{-/-}$ fetal liver cells show ~7-fold increase of apoptosis and significant delayed erythroid differentiation.

*Corresponding author:
E-mail: ramneek1117@yahoo.co.in; Tel. No. +918968144588.

Moreover, when K-ras$^{-/-}$ erythroid progenitors were cultured *in vitro*, there is a significant delay in erythroid differentiation but little increase in apoptosis. In K-ras$^{-/-}$ fetal liver cells, Epo- or stem cell factor (SCF) - dependent Akt activation was greatly reduced whereas other pathways including Stat5 and p44/p42 MAP kinase were activated normally. This indicates that K-ras is the major regulator of cytokine-dependent Akt activation in erythropoiesis *in vivo*.

Importantly, oncogenic mutations in ras genes frequently occur in patients with myeloid disorders and in these patients, erythropoiesis is often affected. Overexpression of oncogenic H-ras in purified mouse primary fetal liver erythroid progenitors blocks terminal erythroid differentiation and supports Epo-independent proliferation. Three major pathways are abnormally activated by oncogenic H-ras: Raf/ERK, PI3-kinase/Akt and RalGEF/RalA. However, only constitutive activation of the MEK/ERK pathway alone could recapitulate all of the effects of oncogenic H-ras expression in blocking the erythroid differentiation and inducing Epo independent proliferation. There are reports that all effects of oncogenic H-ras expression in primary erythroid cells were blocked by the addition of a specific inhibitor of MEK1/2, allowing the normal terminal erythroid proliferation and differentiation. Taken together, the interruption of constitutive MEK/ERK signaling is a potential therapeutic strategy to correct impaired erythroid differentiation in patients with myeloid disorders. But to avoid problems due to oncogenic Ras overexpression, there is a need to study primary erythroid progenitors in which oncogenic Ras is expressed from the endogenous Ras promoter. Expression of oncogenic K-ras is induced using a rtTA/TetO-cre system for a short period of time, and oncogenic K-ras signaling should be assessed in highly purified primary erythroid progenitors. The initial focus should be on the signaling pathways constitutively activated by endogenous oncogenic K-ras and hyperactivated in response to cytokine stimulation. More importantly, the consequences of abnormal oncogenic K-ras signaling in erythroid cells should be evaluated at both the cellular and gene transcriptional levels.

There is sufficient evidence that Lnk-deficient mice have elevated numbers of erythroid progenitors, and that splenic CFU-e progenitors are hypersensitive to Epo. Lnk$^{-/-}$ mice also exhibit superior recovery after erythropoietic stress. In addition, Lnk deficiency results in enhanced Epo-induced signaling pathways in splenic erythroid progenitors. Conversely, Lnk overexpression inhibits Epo-induced cell proliferation. In primary culture of fetal liver cells, Lnk overexpression inhibits Epo-dependent erythroblast differentiation and induced apoptosis; Lnk blocks all three major signaling pathways, Stat5, Akt, and MAPK, induced by Epo in primary erythroblasts. It seems that the Lnk SH2 domain is essential for its inhibitory function, whereas the conserved tyrosine near the C-terminus and the PH domain of Lnk are not critical. Thus Lnk, through its SH2 domain, negatively modulates EpoR signaling by attenuating JAK-2 activation, and regulates Epo-mediated erythropoiesis. There is a need to determine the novel molecular mechanism by which Lnk inhibits signaling from the EpoR and other cytokine receptors.

All changes in gene expression that occur during terminal proliferation and differentiation of purified fetal liver erythroid cells should be assessed. This may involve assay of mRNAs by hybridization to DNA gene microarrays ("gene chips") or immunoprecipitation of chromatin with antibodies specific for transcription factors, followed by hybridization of the recovered DNA to a genomic DNA microarray. This will help determine all of the genes that have critical erythroid-important transcription factors bound to their promoter/enhancer segments. Initial studies may focus on transcriptional activation of Stat5 but other factors also need to be investigated. There is a need to understand how the complex pattern of gene expression during erythroid development is controlled by transcription factors activated by signal transduction pathways downstream of the EpoR.

Also, the role of integrins in terminal proliferation and differentiation of purified fetal liver erythroid cells needs to be assessed, since adhesion of these progenitors to fibronectin is essential for normal erythroid developers. Both a4ß1 and a5ß1 integrins are present on erythroid progenitors, and that a4ß1 and a5ß1 integrins support binding of erythroid cells to different fibronectin domains. There are reports that loss of both a4ß1 and a5ß1 integrins during erythroid differentiation parallels the loss of adhesion of erythroid cells to fibronectin. So, there is a need to investigate the signal transduction pathways and transcriptional changes mediated by each of these integrins and also determining the pattern of integrin expression of purified hematopoietic stem cells.

Epo prevents neuronal death during ischemic events in the brain and in neurodegenerative diseases. The molecular mechanisms of this protection are incompletely understood. Using differentiated human neuroblastoma cells the antiapoptotic activity of Epo has been confirmed and there are reports that Epo activates both the Stat5 and PI-3 kinase/ AKT signaling pathways. Some Studies using expression of chimeric mutant EpoRs able to activate neither or only one of these pathways showed that activation of both is required for EpoR activation to prevent neuronal death. So, there is a need to study apoptosis of primary adult neuronal cells genetically engineered to lack the Epo receptor. Once it is identified how Epo prevents neuronal cell death in the brain, it could lead to a novel clinical application of Epo for limiting brain damage due to stroke or neurodegenerative diseases.

Additionally, Emphasis should be on to develop *in vitro* culture system for erythroid progenitors into an

assay for genotoxicity. Assays that predict toxicity are an essential part of drug development and many drugs fail in phase I clinical trials; therefore, there is a demand for models that can better predict human responses. The mouse *in vivo* micronucleus (MN) assay is a robust toxicity test that assesses the genotoxic effect of drugs by detecting chromosome fragments that remain in the reticulocyte after enucleation; an *in vitro* correlate to this assay might allow extension to human cells and thus better predictive power in drug development. The first steps in developing a toxicity assay could be the adaptation of our *in vitro* erythropoiesis culture system to induce optimized erythropoietic growth from Lin- populations from adult BM, and demonstrating that exposure to genotoxicants induces MN-formation in this culture system. Some researchers have shown that the addition of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) to this culture system induces a global cytotoxic response and concomitant decreases in erythropoietic differentiation and increases in MN-formation. The increase in MN production in the presence of BCNU provides a clear signal of the clastogenic mechanism that likely induced the overall hematopoietic toxicity.