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Modulators of Erythropoiesis: Emerging Therapies For Hemoglobinopathies And Disorders Of Red Cell Production

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JAK2 and disorders associated with chronic stress erythropoiesis

Erythropoiesis is a tightly regulated process that includes cytokine signaling and cell-cell interactions in the specialized niche called erythroblastic islands (1-3). Due the essential role of erythrocytes in oxygen delivery, erythropoiesis can effectively respond and adapt quickly to changes in tissue oxygen tension. This is mediated primarily by EPO (4-6). EPO signals through EPOR (7-9). One of the main factors activated by EPO/EPOR interaction is the cytoplasmatic kinase JAK2 (10-12). Through auto and crossphosphorylation events, JAK2 activates STAT5 and parallel signaling pathways (13, 14). STAT5 migrates to the nucleus activating genes necessary for proliferation, differentiation, and survival of erythroid progenitors. The crucial role of EPO, EPOR, JAK2 and STAT5 has been revealed by knocking out these factors in mice. Absence of each one of these molecules resulted in a lethal anemia during fetal development (12). In particular, phosphorylation of STAT5 is essential for basal erythropoiesis and for its acceleration during hypoxic stress (stress erythropoiesis) (15). Some of the conditions in which the JAK2/STA5 pathway is chronically activated and erythropoiesis is accelerated are polycythemia vera and β-thalassemia (3, 16, 17).

The most recurrent mutation in JAK2, (JAK2 V617F), is associated with myeloproliferative neoplasms (MPNs). This mutation is associated with constitutive phosphorylation of JAK2 and EPO hypersensitivity (18-23). JAK2 inhibitors such as Ruxolitinib, LY2784544 and SAR302503 are presently tested or utilized to treat myelofibrosis, essential thrombocythemia and polycythemia vera (24-26). In particular polycythemia vera, one of the MPNs, is characterized by increased production of erythroid progenitors, erythrocytes and splenomegaly (16, 27-29). In β-thalassemia, hypoxia leads to high levels of EPO in circulation and, in turn, increased erythroid proliferation (16, 27-29). Thus, even in absence of JAK2 mutations, the activity of JAK2 is enhanced, leading to increased proliferation and decreased differentiation of erythroid progenitors (chronic stress erythropoiesis, Figure 1A). This causes a net increase in the number of erythroid progenitors, leading to EMH and

splenomegaly (16, 27-29). Splenomegaly, in turn, increases sequestration of red blood cells, exacerbating the anemia and IE (16, 27-33).

Potential use of JAK2 inhibitors in β-thalassemia

IE is the hallmark of a group of anemias characterized by cell death associated with increased proliferation and decreased differentiation of erythroblasts, ultimately leading to an expansion in the number of erythroid progenitors but with suboptimal or absent production of normal red blood cells (16). The discovery that JAK2 plays an important role in the progression and exacerbation of IE suggests that drugs inhibiting JAK2's activity could mitigate IE (Figure 1B) and reverse splenomegaly. In fact, in preclinical studies it has been shown that a JAK2 inhibitor dramatically decreased the spleen size and modulated the IE (16, 27-33). Based on these observations, use of JAK2 inhibitors could be utilized to reverse splenomegaly, thereby avoiding the need for splenectomy. Ideally, this could also be helpful to reduce the rate of blood transfusions leading to an improved management of anemia and iron overload (16, 27-33).

Activins, members of the TGF-β family signaling

Activins are soluble ligands that, along with bone-morphogenetic-proteins (BMPs), growth-and-differentiation-factors (GDFs) belong to a large group of proteins called transforming growth factor-β (TGF-β) family. The most well characterized activins are homo- or hetero-dimeric structures composed of two very similar β-chains, A or B. Inhibins, on the other hand, are heterodimers composed of α- and β-chains (from activins), and antagonize activins and BMP signaling. Generally, ligands of TGF-β family are synthesized from a common precursor with a prodomain that can determine a ligand's activity and localization (34).

The TGF-β family signal through 7 different type I and 5 different type II transmembrane serine/threonine kinase receptors (35). Type I receptors (activin receptor-like kinases) ALK2, ALK4 and ALK7 (also indicated as ACVR1, ACVR1B, ACVR1C, respectively) and Activin receptor IIA (ActRIIA) and ActRIIB (also indicated as ACVR2A, ACVR2B) are typically the mediators of activins effect (36). ActRIIs are shared by some of the BMPs and GDFs (37). Activin signaling is carried out through formation of a ternary complex between the ligand, the type II and the type I receptors, which ultimately phosphorylates SMAD proteins (35, 36). SMADs multimerize and these complexes translocate to the nucleus and regulate gene expression in concert with other transcription factors. Follistatin (FST) and FST-related protein (FRP) bind extracellularly to activins and other related TGF-β ligands, controlling their signaling and availability (38-40). FST inhibits activin by hiding 1/3 of its residues as well as type I and II receptors binding sites (38-40). Mice with a disrupted FST gene have musculoskeletal and skin abnormalities, while mice with the gene encoding FRP (follistatin-related gene or FLRG) deleted show disregulated glucose metabolism and fat homeostasis (41).

Activins are expressed in various tissues and have a broad range of activities that regulate:

- Gonadal function
- Hormonal homeostasis
- Growth and differentiation of musculoskeletal tissues
- Growth and metastasis of cancer cells
- Proliferation and differentiation of embryonic/hematopoietic stem and erythropoietic cells
- Higher brain function

Activin activities are involved in the etiology and pathogenesis of several diseases (42). Dysregulation of activin signaling has been associated with many malignant disorders as well as diseases affected by anemia. For these conditions the inhibition of activin signaling represents an interesting therapeutic approach (42).

Cancer-related anemia and ineffective erythropoiesis

Anemia is a condition that affects hematological malignancies like multiple myeloma and myelodysplastic syndromes (MDS). MDS encompasses a heterogeneous group of closely related clonal hematopoietic disorders characterized by a marrow with impaired maturation (dysmyelopoiesis) and peripheral blood cytopenias, resulting from ineffective blood cell production (43), and develops when a clonal mutation predominates in the bone marrow, suppressing healthy stem cells. Anemia can also be caused by myelosuppressive chemotherapy. Anemia is also observed in some tumors in absence of chemotherapy treatment and it can be consider a prognostic factor of reduced survival. IE also leads to anemia and it is the hallmark of a group of diseases such as β-thalassemia. Individuals with these anemias have markedly elevated EPO levels that result in massive erythroid expansion in the hypercellular marrow (44, 45). When abnormalities in the red cells lead to their intramedullary demise, erythropoiesis is ineffective and leads to enhanced intestinal iron absorption, and resultant tissue iron loading and toxicity. The thalassemia syndromes (both α- and β-thalassemia) are the most frequent conditions associated with IE and result from diminished production of α- or β-globin chains respectively, the two subunits of the hemoglobin A (adult) molecule (27, 46-48). Due to IE, patients develop several co-morbidities, including iron overload and bone abnormalities. Another group of conditions characterized by IE are congenital and acquired sideroblastic anemias. In these conditions, the bone marrow produces sideroblasts characterized by granules of iron accumulated in perinuclear mitochondria, forming a “ring” around the erythroblast nucleus. These ringed sideroblasts fail to differentiate into healthy erythrocytes (49). Acquired sideroblastic anemias may be caused either by a genetic disorder or indirectly as part of the MDS (50). Other erythroid disorders that might benefit from ameliorating the IE are congenital dyserythropoietic anemias, chronic pernicious anemia and hereditary spherocytosis (51).

Individuals affected by thalassemia and MDS receive blood transfusion to compensate for the anemia, but with time several co-morbidities develop, including iron overload, bone abnormalities and, in MDS patients, progression to acute myeloid leukemia (AML). Although improved transfusion and iron chelation treatments over the past 2 decades or so have reduced morbidity and improved the life expectancy of patients, they do not provide a definitive cure and lack the ability to correct IE. Therefore, alternative pharmacological therapies that target the direct recovery of terminal erythroid differentiation are urgently needed.

The use of Erythropoietin (EPO) and its derivatives, also referred to as erythropoiesis stimulating agents or ESAs, to treat cancer-related anemia can be controversial as it has been speculated it might aggravate tumor progression (52). In addition, ESAs have been used for diseases characterized by anemia caused by IE but with limited benefits (53). Therefore new pharmacological treatments with different mechanism of actions are needed. In this perspective the use of molecules that can target EPO-unrelated pathways, like activin signaling, might be potential candidate to ameliorate anemia.

Effect of activin signaling in bone

In vitro and *in vivo* data on activin signaling are discordant. Mice injected with activin A show increase of bone formation and bone strength, although in rats similar results were

reproduced using inhibin A (54). In peri- and postmenopausal women follicle-stimulating hormone (FSH) seems to indirectly exert its anabolic effect on bone through an ovarian mediator (possibly inhibin A) (55). Withdrawal of inhibin A could ultimately be related to bone loss observed in peri and postmenopause. FST has been shown to have an opposite effect (56) indicating that FST and inhibins do not operate as analogue in the bone context. In relationship to metastatic bone progression elevated levels of circulating activin A seem to be a prognostic factor. In fact, breast and prostatic cancer patients with bone metastasis present higher levels of activin A than non metastatic patients (57). A similar observation was made for patients with multiple myeloma, where high activin levels correlate with extensive bone involvement and lower survival rate (58). Moreover, in vitro studies have shown that multiple myeloma cells stimulate stromal cells to produce activin, which in turn leads to inhibition of osteoblasts differentiation *in* vitro (59).

Effect of activin signaling in cancer

In vivo studies showed that lack of inhibin in transgenic mice causes gonadal and adrenal tumorigenesis, indicating that these activin repressors are important tumor repressor in these tissues (60). Overexpression of FST in these mice does not reduce tumor incidence but modulate tumor progression and reduces the tumor-cachexia like syndrome associated with high levels of activin (61). As for the liver, FST adenoviral-induced over expression causes hepatocytes hyper proliferation (62). The opposite effect is observed when over expression of FST is induced in small cell lung cancer cells, which seem to produce a reduction of experimental metastases in various organs in NOD-SCID mice (41). Compared to transgenic activin deficient mice, FST deficient mice die before birth due to compromised development of growth, skin, muscle and skeletal development. Therefore FST operates on many signaling pathway in addition to the activin-related ones. The function of FST and activin in different tissues can differ vastly, therefore their levels as indicator of tumor development, progression and metastases might be challenging, although certainly impactful (63).

Effect of activin signaling in hematopoiesis and erythropoiesis

Members of the TGF-β family are also key regulators of human hematopoiesis, modulating various cellular responses such as proliferation, differentiation, migration and apoptosis. Activin expression can be detected in bone marrow cells, including eryhthroid cells. Recombinant activin induces cellular shrinking and nuclear condensation of $CD34^+$ cells during *in vitro* differentiation. This process is reverted by addition of FST related, FRP, BMP2 and BMP4 (64, 65).

Activin A and bone morphogenetic proteins BMP2 and BMP4, alone or in combination, have been shown to have a role in the regulation of erythropoiesis in various models (66, 67).

The biological function of activin A, BMP2 and BMP4 was assessed measuring clonogenic potential in colony CFC assay of human $CD34^+$ cells isolated from either mobilized peripheral blood or bone marrow (68). Activin was found to increase the number of both late erythroid burst-forming unit (BFU-E) and erythroid colony-forming unit, (CFU-E). This observation was confirmed *in vivo* through injection of activin in anemic (phlebotomized) and normal mice (69). On the other hand, BMP2 was found to increase the number of early BFU-E. Cells treated with activin A showed a significant decrease in nuclear size, a phenomenon associated with maturation of erythroid cells. In comparison to activin A alone, addition of BMP4 induces an inhibitory effect in the number of late BFU-E and CFU-E, and increases nuclear size (70-72). This suggests that BMP4 facilitates differentiation of the erythroid progenitors before they loose their ability to form colonies and their nuclear size

start shrinking. BMP2 antagonizes the effect of activin A on nuclear size reduction (64, 65). Therefore BMP molecules may have different effect on the activin A-mediated biological activities.

In hematopoiesis FST has an inhibitory effect on activin (65). Both FST and FRP neutralize also GDF11 and myostatin (73). Both repressors interfere with the ability of activin to induce nuclear size reduction and increase counts of early BFU and CFU, and also inhibit BMP2-induced early BFU proliferation. BM stromal cells are among the tissues that produce at steady state the highest levels of FLRG transcripts (74). They also produce activin and FST (74). Among hematopoietic lineages, monocytes have the highest level of expression of FLRG (74). Expression of FLRG in erythroid cells is mostly observed in immature cells while FST is expressed at highest levels in most mature erytrhoid cells (64, 74, 75). The expression of both FST and FLRG transcripts are induced by activin A as well as TGF-β, indicating that these two repressors participate in a negative feedback loop that regulates activin signaling. Additional regulation of activin and TGF-β ligands signaling pathway occurs intracellularly via the SMAD inhibitory protein 6 and 7 (76, 77). These proteins induce ubiquitin-dependent degradation of SMAD 2, 3 and 4 (76-78).

Therapeutic interventions that target activin signaling

Several strategies have been developed to hinder the dysregulation of activin signaling. These include the use of:

- Small molecules that inhibit type 1 receptors
- Antibodies that inhibit the interaction between activins and their receptors
- Activin mutant proteins that bind to ActIIRA but block subsequent ternary interaction with receptor type 1, ALK4
- Chimeric polypeptides that sequester activin but not GDF8 or 11
- Ligand traps that act in a similar fashion to follistatin/FSRG

Currently the approaches with highest clinical impact are those based on small molecules that inhibit type 1 receptors or ligand trap soluble molecules that sequester ligands of ActRIIA and B, like ACE-011 and ACE-536.

Small molecules targeting type 1 receptors

These molecules have been developed mainly to target ALK5, the type 1 receptor of TGF-β, with specific focus on cancer treatment. Most molecules developed have shown broader range of affinity and target not only ALK5 but also ALK4 and 7 (42). Therefore they are considered inhibitors or activin and TGF-β as well. Because of the broader mechanism of action they are still evaluated and their use in clinical trial is subject to further screening. Among these molecule LY-2157299, a potent inhibitor of ALK 5 has now been used in 5 different clinical trials that include phase I-II cancer and metastatic-cancer as well MDS studies (42).

Zhou and colleagues showed that SMAD7 expression (an inhibitor of the SMAD pathway activated by TGF- β (79) is significantly reduced in bone marrow-derived $CD34^+$ cells isolated from low-grade MDS patients compared to healthy subjects. This downregulation induces more sensibility to TGF-β stimulation (77). Therefore the pathways associated with TGF-β are hyper activated, likely affecting cell differentiation and proliferation. This is mostly mediated through ALK5. In fact, when LY-2157299 is given to a mouse model of MDS, this ameliorates RBC synthesis, with increased hematocrit and hemoglobin levels (77). MDS bone marrow cells exhibit poor hematopoietic colony formation. Treatment of

mononuclear cells isolated from low-grade MDS patients with the ALK5 inhibitor increased both erythroid and myeloid colony numbers, which points to a high therapeutic potential of ALK5 inhibition by LY-2157299 in MDS patients that do not have increased blast counts (77).

Pre- and clinical studies with ACE/RAP-011

ACE-011 is a receptor fusion protein that functions as a soluble trap that sequesters ligands of the ActRIIA. It is a truncated form of the extracellular domain of the human ActRIIA combined with the Fc of the human immunoglobulin IgG1. In the mouse ortholog, RAP-011, the extracellular domain of the human ActRIIA is combined with the Fc of the mouse immunoglobulin IgG2a.

Several studies have shown beneficial effects of RAP-011 in mouse models of various conditions. RAP-011 was able to ameliorate anemia induced by chemotherapy treatment of mice with paclitaxel (80). RBCs were also elevated after treatment of normal mice with the drug.

Reduction of osteolytic lesions and number of multiple myeloma tumor cells, in addition to strengthening of the bones was observed in a humanized mouse model of multiple myeloma (59). RAP-011 also reduced osteolytic lesions and metastatic progression in a breast cancer mouse model, prolonging mouse survival (81).

Two clinical studies (82, 83) in healthy volunteers have shown that in postmenopausal women, a single administration (I.V. or S.C., up to 3mg/kg) of ACE-011

- Reduces FSH serum levels
- Increases levels of bone formation biomarkers like bone-specific alkaline phosphatase (BSAP);
- Increases RBC, hematocrit and hemoglobin levels (leaving white cells and platelet counts unchanged) in a dose dependent manner and in a fashion that differs from the mechanism of action of ESAs.

The half-life of the drug is 24-32 days. The increase of RBC count suggests that one or more ligands of ActRIIA might act as a negative regulator in normal erythropoiesis.

Iancu-Rubin and colleagues investigated the mechanisms behind the beneficial effect of ACE-011 on red blood cells production *in vitro* (84). Although they couldn't detect a direct effect on erythroid differentiation of human $CD34^+$ cells, they found that ACE-011 attenuates the inhibitory effect of bone marrow conditioned media on cell differentiation. Therefore the effect of the drug could be attributed to an indirect modulation of the microenvironment in which $CD34^+$ cells differentiate rather than a direct action on the erythroid precursors.

Because of its ability to ameliorate anemia, ACE-011 has been used in clinical trials for multiple myeloma patients (NCT00747123, completed and NCT01562405, currently recruiting patients). Additionally, the drug is under investigation in several ongoing trials that are recruiting patients for:

- The treatment of anemia in low- or intermediate-1 risk MDS or Non-proliferative Chronic Myelomonocytic Leukemia (CMML) (NCT01736683)
- Testing safety and efficacy in adults with transfusion dependent Diamond Blackfan Anemia (NTC01464164)

- Testing safety and tolerability in adults with β-thalassemia (NTC01571635).

Pre- and clinical studies with ACE/RAP-536

As mentioned previously, several members of the TGFβ-superfamily are involved in regulating erythropoiesis. ACE-536 (and its mouse ortholog RAP-536) is a modified activin type IIB receptor (Act-RIIB) fusion protein that does not inhibit activin A induced signaling, but inhibits signaling induced by other members of the TGF-β superfamily. While EPO increases proliferation of erythroid progenitors, RAP-536 promotes maturation of terminally differentiating erythroblasts. In thalassemic mice ($Hbb^{th1/th1}$), RAP-536 ameliorates hematological parameters as well as co-morbidities that develop as a consequence of the erythroid hyperplasia (85).

In NUP98-HOXD13 (NHD13) MDS mice, RAP-536 corrects IE and normalizes myeloid:erythroid ratio, retarding progression to leukemia (85, 86). In both mouse models RAP-536 rescued disease phenotypes by promoting terminal erythroid differentiation, thereby enhancing effective erythropoiesis. Cell cycle analyses of bone and splenic erythroblasts isolated from mice treated with RAP-536 showed decrease in S-phase and increase in G1/2 phases compared to placebo treated animals. At 72hr after treatment with RAP-536 a decrease of basophilic and increase of ortho- and poly-chromatic erythroblasts and reticulocytes is observed, with a resultant increase in hemoglobin as compared to placebo treated wild type mice. Altogether, these observations suggest that the mechanism of action of these trap ligands works through acceleration of the differentiaton of the erythroid precursors, normalizing the ratio between proliferation and differentiation of the erythoid precursors under condition of IE (Figure 1C). In addition, these preclinical data have provided a rationale for clinical studies of the human ortholog ACE-536, which is in two European phase-II clinical trials, for the treatment of β-Thalassemia intermedia (NCT01749540) and MDS (NCT01749514).

Conclusions

Use of new compound such as inhibitors of JAK2 or TGF-β-like molecules might soon revolutionize the treatment of β-thalassemia and related disorders. However, this will require a careful optimization noting the potential for off-target immune suppression for JAK2 inhibitors and the lack of mechanistic insights for the use of the ligand trap soluble molecules that sequester ligands of ActRIIA and B.

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Key points

- Increased proliferation of erythroid progenitors is a feature common to many pathological conditions associated with anemia or an excessive production of red cells
- Chronic stress erythropoiesis (associated with ineffective erythropoiesis (IE) and anemia or erythrocitosis) leads to severe comorbities that aggravate patients condition
- In the context of tumorigenesis, the level of JAK2 activity or activin seems to correlate directly with disease progression
- Development of drugs that target Epo dependent or independent pathways, like JAK2 or activin signaling, can be beneficial in hemoglobinopathies and other erythroid disorders

Synopsis

EPO serves as the master regulator of erythropoiesis. EPO signals through the erythropoietin receptor (EPOR), activating the cytoplasmatic kinase JAK2. Activation of JAK2 lead to activation of the signal transductor and activator of transcription Stat5 a and b, whose levels of expression modulate steady state vs. stress erythropoiesis. Superphysiological levels of JAK2 activity are associated with increased proliferation of erythroid progenitors, extramedullary hematopoiesis (EMH) and splenomegaly. JAK2 inhibitors such as Ruxolitinib, LY2784544 and SAR302503 are presently tested or utilized to treat myelofibrosis, essential thrombocythemia and polycythemia vera; the same inhibitors might have a beneficial effect in preventing or reversing splenomegaly and EMH in several hemoglobinopathies.

Activin signaling occurs in many organs and supports physiological processes via interaction of ligands of the transforming growth factor-β (TGF-β) family and a network of downstream key players. Dysregulation of activin signaling is observed in conditions that affect several processes, including hematopoiesis and erythropoiesis. Clinical trials based on molecules that interfere with the pathological hyperactivation of activin signaling mediated by activin type II and I receptors as well as other co-players have been initiated. Among several candidates ACE-011, ACE-536, and LY-2157299 are some of the most successful drugs developed for several pathologies. These drugs have shown to ameliorate the anemia that underlies most of the treated conditions and that ultimately determines patients' prognosis.

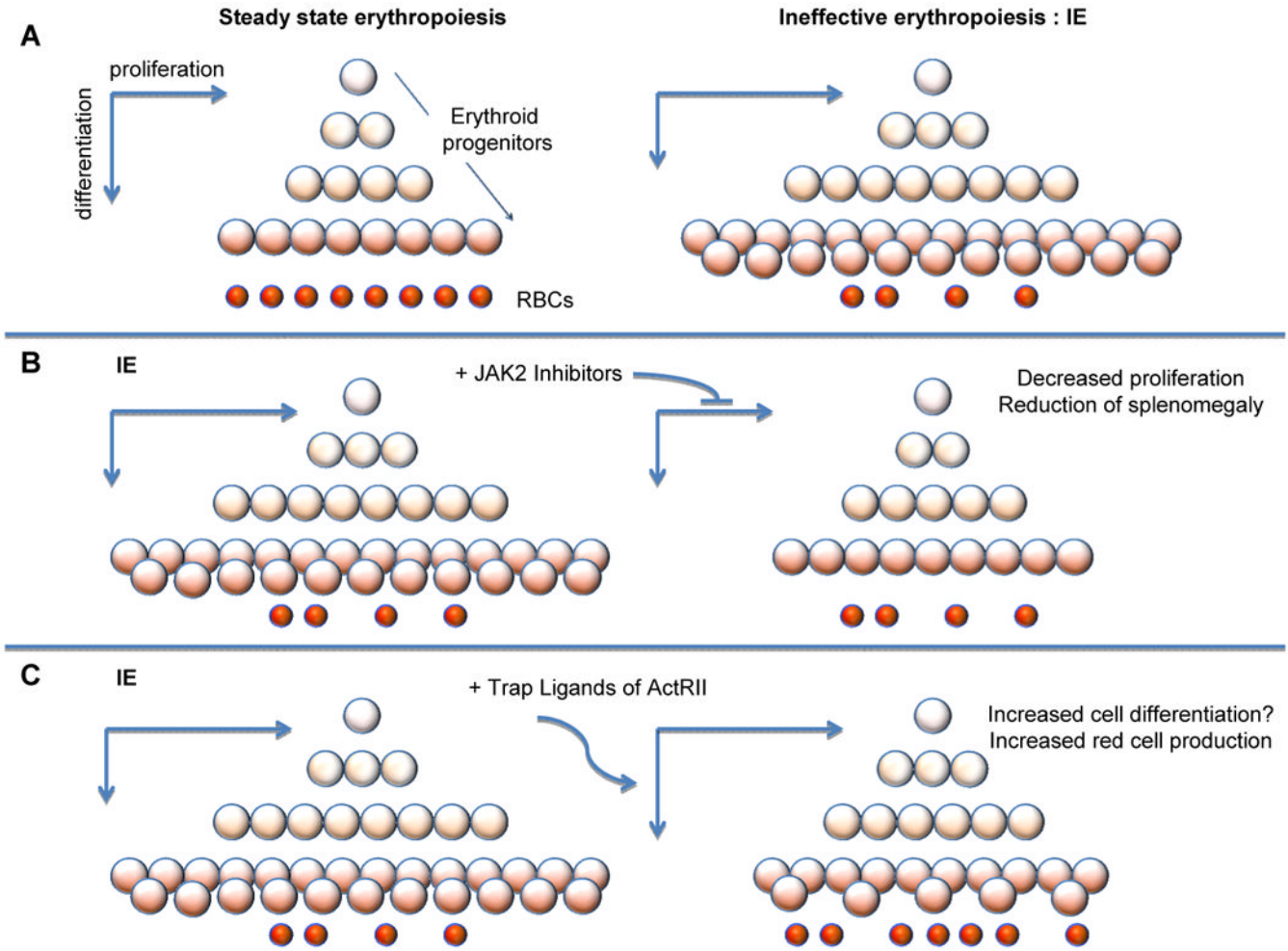

Figure 1. Schematic representation of physiological and ineffective erythropoiesis (IE); effect of JAK2 inhibitors and trap ligands of activin II receptors. A

Compared to normal (left), IE (right) is characterized by the expansion of immature erythroid cells that fail to differentiate into red blood cells. This phenomenon is responsible for the spleen enlargement (splenomegaly) observed in conditions characterized by IE. **B**. Inhibiton of the JAK2 pathway reduces spleen enlargement by interfering with extensive proliferation of immature erythroid cells. **C**. Trap ligands of the activin receptors II ameliorate anemia in IE possibly by normalizating the ratio between proliferation and differentiation of immature erythroid cells.