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(54) Title: METHODS AND COMPOSITIONS FOR MODULATING ERYTHROPOIESIS

(57) Abstract: In certain aspects, disclosed herein are novel compositions and methods related to either the enhancement or inhibition of erythropoiesis that are useful, for example, in the treatment of anemia or erythrocytosis.

METHODS AND COMPOSITIONS FOR MODULATING ERYTHROPOIESIS

RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Application No. 62/394570 filed September 14, 2016, which is hereby incorporated by reference in its entirety.

GOVERNMENT INTEREST

This invention was made with Government support under National Institutes of Health Grant R01DK100915. The Government has certain rights in the invention.

BACKGROUND

To date, there are only a small number of erythropoiesis stimulating agents available for therapy, limited to derivatives of the hormone erythropoietin (Epo), iron supplements, and corticosteroids, agents to which resistance or contraindication is not uncommon. These agents are used in anemia of various etiologies including kidney disease, myelodysplastic syndrome, and anemia associated with chronic inflammatory disease. For anemia associated with cancer, where Epo was previously used effectively to treat anemia, it is now contraindicated, since it was found to increase mortality. There is also a shortage of erythropoiesis suppressing agents, a class of agents that is not generally used clinically but could be useful in the treatment of the premalignant syndrome Polycythemia Vera or in inherited primary erythrocytosis syndromes.

The shortage of erythropoiesis stimulating and suppressing agents reflects the paucity of tools available today for the study of early erythropoiesis. There have been no strategies that systematically identify the entire cellular and molecular trajectory of the early erythroid lineage.

SUMMARY

In certain aspects, disclosed herein are novel compositions and methods related to either the enhancement or inhibition of erythropoiesis that are useful, for example, in the treatment of anemia or erythrocytosis.

In some aspects, the methods provided herein relate to the stimulation of erythropoiesis in a subject (*e.g.*, a human subject). In some embodiments, provided herein is a method of stimulating erythropoiesis in a subject with anemia comprising administering to the subject an agent that binds to MST1r and/or induces or inhibits MST1r activity (*e.g.*, a

MSP ligand protein or a fragment thereof). In some embodiments, provided herein is a method of stimulating erythropoiesis in a subject with anemia comprising administering to the subject an agent that binds to IL-17ra (*e.g.*, a IL-17a protein or a fragment thereof, a IL-17f protein or a fragment thereof, or a heterodimeric IL-17a/f ligand). In some embodiments, provided herein is a method of stimulating erythropoiesis in a subject with anemia comprising administering to the subject an agent that inhibits the binding of WNT5a to RYK and/or inhibits WNT5a and/or RYK expression or activity.

In certain aspects, the methods provided herein relate to the inhibition of erythropoiesis in a subject (*e.g.*, a human subject). In some embodiments, provided herein is a method of inhibiting erythropoiesis in a subject with erythrocytosis comprising administering to the subject an agent that that inhibits the binding of MSP to MST1r and/or inhibits MST1r and/or MSP expression or activity. In some embodiments, provided herein is a method of inhibiting erythropoiesis in a subject with erythrocytosis comprising administering to the subject an agent that binds RYK and/or induces RYK activity (*e.g.*, a WNT5a protein or a fragment thereof). In some embodiments, provided herein is a method of inhibiting erythropoiesis in a subject with erythrocytosis comprising administering to the subject an agent that that inhibits the binding IL-17a to IL-17ra and/or IL-17a expression or activity.

In certain aspects, provided herein are agents (*e.g.*, polypeptide agents, small molecule agents, antibody agents and/or inhibitory nucleic acid agents) useful in the methods disclosed herein, as well as pharmaceutical compositions comprising such agents and methods of identifying such agents.

In certain aspects, provided herein are methods of expanding or propagating erythroid progenitor cells *in vitro* comprising supplementing or adding an agent that activates IL-17ra to a cell medium comprising the progenitor cells. In some embodiments, provided herein are methods of expanding or propagating erythroid progenitor cells *in vitro* comprising supplementing or adding an agent that inhibits Ryk and/or Mst1r to a cell medium comprising the progenitor cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 includes 5 panels (Panels a-e). Panel a is schematic depicting single cell RNA expression analysis of hematopoietic progenitor cells (HPC). Panel b is a schematic of the analytical pipeline as described in Example 1. Panel c shows SPRING plots of single cell

trancriptomes. Panel d shows the SPRING plots analyzed for erythroid trajectory. Panel e shows data representing the expression of established erythroid regulators.

Figure 2 includes 7 panels (Panels a-h), which show data representing dynamic patterns in gene expression in early erythropoiesis

Figure 3 includes 5 panels (Panels a-e), which show validation of the SPRING plot and PBA predictions provided herein.

Figure 4 includes 3 panels (Panels a-c). Panel a shows SPRING plots and temporal ordering of gene expression of receptors during erythropoiesis. Panel b show the qPCR validation on fractionated cell populations. Panel c shows the CFUe assay results for the three ligands.

Figure 5 includes 7 panels (Panels a-h), which show data representing synchronization in S phase at the switch from early erythropoiesis to ETD

Figure 6 includes 7 panels (Panels a-g), which show that certain ligands described herein provide modulation of pre-ETD erythroid progenitors.

Figure 7 shows CFUe and BFU-e (early and late) assay results for ligands - MSP, WNT5a, and IL-17a.

Figure 8 shows exemplary nucleic acid and amino acid sequences of MST1r.

Figure 9 shows exemplary nucleic acid and amino acid sequences of MSP.

Figure 10 shows exemplary nucleic acid and amino acid sequences of RYK.

Figure 11 shows exemplary nucleic acid and amino acid sequences of WNT5a.

Figure 12 shows exemplary nucleic acid and amino acid sequences of Il-17ra.

Figure 13 shows exemplary nucleic acid and amino acid sequences of IL-17a.

Figure 14 includes 7 panels (Panels a-f). Panel a shows a flow-cytometric sorting strategy, dividing $Kit^{+}CD55^{+}$ BM cells into gates P1 to P5 based on expression of CD49f (*Itga6*), CD105 (*Eng*), CD41 (*Itga2b*), CD150 (*Slamf1*), and CD71 (*Tfrc*). Freshly sorted cells from each gate were profiled using scRNA-Seq. Panel b shows the sorted subpopulations P1-P5 localized on the Kit+ graph by assigning single cell transcriptomes from each sub-population to their most similar counterparts. The density of projected cells is represented using a heatmap. Panels c through e show colony formation assays in methylcellulose carried out on freshly sorted P1 to P5 subsets and on $Kit^{+}CD55^{-}$ cells. These assays show that P1 and P2 subsets completely define classic CFU-e and BFU-e progenitors. Erythroid colonies were classified as either unifocal (Panel c) or multifocal (Panel d). CFU-

MK=Megakaryocytic colonies, CFU-GM= granulocytic and/or monocytic colonies; CFU-GEMM= mixed myeloid colonies. Results are mean ± SE of two independent sorts. Colony assays were performed in triplicate. Images show erythroid colonies, stained with diaminobenzidine to highlight hemoglobin expression. The P1 population enriches 150-fold for CFU-e, compared with total bone marrow (BM); the P2 population enriches 50-fold, 80-fold and 45-fold for CFU-e, late and early BFU-e, respectively. Panel f is a summary of erythroid colony formation potential in FACS subsets.

Figure 15 has 8 panels (panels a-h). Panel a shows that expression of *Mst1r*, *Ryk* and *Il17ra* peaks at CEP or EEP stage. Panel b shows representative changes in the number of CFU-e colonies formed in methylcellulose in the presence of the cognate ligands MSP, Wnt5a or Il17a to culture media. Error bars show SD of two independent experiments, with four replicates per experiment. Panel c shows representative Epo dose response curve for IL-17a, showing a persistent IL-17a response even at saturating doses of Epo. Panel d shows that the IL-17a response is dependent on IL-17Ra expression, as demonstrated by a loss of response in colonies from IL-17Ra-/- mice. Colonies scored per 500,000 plated bone marrow (BM) cells in the presence of Epo at 0.05 U /ml. Data is mean ± SD of triplicate samples; representative of two independent experiments. Panel e shows that IL-17a also stimulates CFU-e in freshly isolated human bone marrow mononuclear cells. Colonies scored per 85,000 cells plated. Data is mean ± SD of triplicate samples. Panel f shows FACS analysis of fixed and permeabilized cells stained for pStat3 and pStat5 and shows that IL-17a rapidly activates intracellular signaling through these pathways independently of Epo. Freshly isolated mouse BM cells were starved of cytokines for 3 hours, and then stimulated with Epo, IL-17a or both cytokines. FACS profiles on *left* are for baseline (starved) cells (solid histograms), and 60 minutes –post stimulation (line histograms). On the *right* is a time course of the fraction (%) of cells positive for either pStat in the same experiment. Representative of two experiments. Panel g shows a western blot showing IL-17Ra peak expression in P2/EEP cells and dropping in P1/CEP and in the granulocytic branch (which contributes most of the CD55- cells), consistent with the SPRING plots in (Panel a). Panel h shows a summary of effects of growth factor regulators described herein on erythroid output.

Figure 16 has two panels (a-b), and shows the localized gene expression and functional response of the erythroid lineage to stimulation of Mst1, Ryk and IL-17Ra. Panel a shows predicted expression of *Mst1r*, *Ryk* and *Il17ra* ass assessed by` the smoothed

scRNA-Seq gene expression on cells arranged along the erythroid trajectory in basal bone marrow (BM), fetal liver (FL) and endosteal bone marrow (eBM). Panel b shows expression of *Mst1r*, *Ryk* and *Il17ra* in basal BM as measured by RT-qPCR.

DETAILED DESCRIPTION

General

In certain aspects, provided herein are novel compositions and methods for the enhancement or inhibition of erythropoiesis and/or for the treatment of anemia or erythrocytosis.

Disclosed herein are three previously unappreciated growth factor receptors found to regulate erythropoiesis, which were identified by searching for receptors expressed in early erythropoiesis through a single cell RNA-Seq reconstruction of the hematopoietic lineage and validated by in vitro erythroid colony-forming-unit and burst-forming unit assays, in mouse fetal liver and in mouse adult bone marrow.

As disclosed herein, three target receptors were identified that are expressed at high levels in early erythroid progenitors: RYK, MST1r and IL-17ra. The ligands of these receptors, WNT5a, MSP and IL-17a (to RYK, MST1r and IL-17ra respectively), interact with erythropoietin (Epo), an essential erythroid cytokine, to either stimulate or inhibit erythropoiesis, as established by colony formation assays and described below. For example, in the fetal liver, MSP interacting with MST1r was determined to enhance erythropoiesis in the context of low erythropoietin concentrations (*e.g.*, 50 mU/ml or less), but inhibit erythropoiesis in the context of high erythropoietin concentrations (*e.g.*, greater than 100 mU/ml). MSP also demonstrated an inhibitory effect in the bone marrow. As demonstrated herein, WNT5a ligand (through its interaction with RYK) is a consistent and potent inhibitor of all erythroid colony formation in both fetal liver and bone marrow. What is more, as shown herein, IL-17a, through its interaction with IL-17ra, potentiates bone marrow erythropoiesis.

Accordingly, in certain aspects, disclosed herein are novel compositions and methods for the enhancement or inhibition of erythropoiesis and/or for the treatment of anemia or erythrocytosis through the stimulation or inhibition of RYK, MST1r and IL-17ra and their associated ligands, WNT5a, MSP and IL-17a, respectively. Also disclosed herein are novel compositions and methods for the treatment of anemia through the stimulation of IL-17ra by IL-17a, IL-17f, or by heterodimeric IL-17a/f ligands.

In some aspects, disclosed herein are methods of expanding or propagating erythroid progenitor cells *in vitro* comprising supplementing or adding an agent that activates IL-17ra and/or an agent that inhibits Ryk or Mst1r to a cell medium comprising the progenitor cells.

Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

As used herein, the term "*administering*" means providing a pharmaceutical agent or composition to a subject, and includes, but is not limited to, administering by a medical professional and self-administering.

The term "*agent*" is used herein to denote a chemical compound, a small molecule, a mixture of chemical compounds and/or a biological macromolecule (such as a nucleic acid, an antibody, an antibody fragment, a protein or a peptide). Agents may be identified as having a particular activity by screening assays described herein below. The activity of such agents may render them suitable as a "therapeutic agent" which is a biologically, physiologically, or pharmacologically active substance (or substances) that acts locally or systemically in a subject.

The term "*amino acid*" is intended to embrace all molecules, whether natural or synthetic, which include both an amino functionality and an acid functionality and capable of being included in a polymer of naturally-occurring amino acids. Exemplary amino acids include naturally-occurring amino acids; analogs, derivatives and congeners thereof; amino acid analogs having variant side chains; and all stereoisomers of any of the foregoing.

As used herein, the term "*antibody*" may refer to both an intact antibody and an antigen binding fragment thereof. Intact antibodies are glycoproteins that include at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain includes a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. Each light chain includes a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen.

The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system. The term "antibody" includes, for example, monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), single-chain antibodies and antigen-binding antibody fragments. An "*isolated antibody,*" as used herein, refers to an antibody which is substantially free of other antibodies having different antigenic specificities. An isolated antibody may, however, have some cross-reactivity to other, related antigens.

The terms "*antigen binding fragment*" and "*antigen-binding portion*" of an antibody, as used herein, refers to one or more fragments of an antibody that retain the ability to bind to an antigen. Examples of binding fragments encompassed within the term "antigen-binding fragment" of an antibody include Fab, Fab', $F(ab')_2$, Fv, scFv, disulfide linked Fv, Fd, diabodies, single-chain antibodies, NANOBODIES®, isolated CDRH3, and other antibody fragments that retain at least a portion of the variable region of an intact antibody. These antibody fragments can be obtained using conventional recombinant and/or enzymatic techniques and can be screened for antigen binding in the same manner as intact antibodies.

The terms "*CDR*", and its plural "*CDRs*", refer to a complementarity determining region (CDR) of an antibody or antibody fragment, which determine the binding character of an antibody or antibody fragment. In most instances, three CDRs are present in a light chain variable region (CDRL1, CDRL2 and CDRL3) and three CDRs are present in a heavy chain variable region (CDRH1, CDRH2 and CDRH3). CDRs contribute to the functional activity of an antibody molecule and are separated by amino acid sequences that comprise scaffolding or framework regions. Among the various CDRs, the CDR3 sequences, and particularly CDRH3, are the most diverse and therefore have the strongest contribution to antibody specificity. There are at least two techniques for determining CDRs: (1) an approach based on cross-species sequence variability (*i.e.*, Kabat et al., Sequences of Proteins of Immunological Interest (National Institute of Health, Bethesda, Md. (1987), incorporated by reference in its entirety); and (2) an approach based on crystallographic studies of antigen-antibody complexes (Chothia et al., Nature, 342:877 (1989), incorporated by reference in its entirety).

As used herein, the term "*humanized antibody*" refers to an antibody that has at least one CDR derived from a mammal other than a human, and a FR region and the constant region of a human antibody. A humanized antibody is useful as an effective component in a therapeutic agent since antigenicity of the humanized antibody in human body is lowered.

The term "*isolated polypeptide*" refers to a polypeptide, in certain embodiments prepared from recombinant DNA or RNA, or of synthetic origin, or some combination thereof, which (1) is not associated with proteins that it is normally found with in nature, (2) is isolated from the cell in which it normally occurs, (3) is isolated free of other proteins from the same cellular source, (4) is expressed by a cell from a different species, or (5) does not occur in nature.

The term "*isolated nucleic acid*" refers to a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, which (1) is not associated with the cell in which the "isolated nucleic acid" is found in nature, or (2) is operably linked to a polynucleotide to which it is not linked in nature.

As used herein, the term "*monoclonal antibody*" refers to an antibody obtained from a population of substantially homogeneous antibodies that specifically bind to the same epitope, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method.

The terms "*polynucleotide*", and "*nucleic acid*" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The

sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified, such as by conjugation with a labeling component. The term "recombinant" polynucleotide means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which either does not occur in nature or is linked to another polynucleotide in a non-natural arrangement.

The phrase "*pharmaceutically-acceptable carrier*" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body

As used herein, "*specific binding*" refers to the ability of an antibody to bind to a predetermined antigen or the ability of a polypeptide to bind to its predetermined binding partner. Typically, an antibody or polypeptide specifically binds to its predetermined antigen or binding partner with an affinity corresponding to a K_D of about 10^{-7} M or less, and binds to the predetermined antigen/binding partner with an affinity (as expressed by K_D) that is at least 10 fold less, at least 100 fold less or at least 1000 fold less than its affinity for binding to a non-specific and unrelated antigen/binding partner (*e.g.*, BSA, casein).

As used herein, the term "*subject*" means a human or non-human animal selected for treatment or therapy.

The phrases "*therapeutically-effective amount*" and "*effective amount*" as used herein means the amount of an agent which is effective for producing the desired therapeutic effect in at least a sub-population of cells in a subject at a reasonable benefit/risk ratio applicable to any medical treatment.

"*Treating*" a disease in a subject or "*treating*" a subject having a disease refers to subjecting the subject to a pharmaceutical treatment, *e.g.*, the administration of a drug, such that at least one symptom of the disease is decreased or prevented from worsening.

Erythropoiesis Modulating Proteins

In certain embodiments, the compositions and methods provided herein relate to three erythropoiesis-modulating receptors, MST1r, RYK and IL-17ra, as well as their associated ligands, MSP, WNT5a and IL-17a, respectively. Additional ligands for IL-17a include IL-17f and heterodimeric IL-17a/f ligands.

MST1r (macrophage stimulating 1 receptor/ RON kinase) is a receptor tyrosine kinase (RTK) expressed in multiple tissues and tumors. As disclosed herein, engagement of MST1r by its ligand MSP inhibits bone marrow erythropoiesis. It also stimulates fetal liver erythropoiesis in the presence of low Epo concentrations (Epo at less than 100 mU/ml). Thus, agents that inhibit MST1r (*e.g.*, an agent that directly disrupts the interaction between MST1r and MSP, such as an MST1r-specific or MSP-specific antibody and/or an agent that inhibits MST1r and/or MSP expression, such as an MST1r-specific and/or MSP-specific inhibitory nucleic acid) can enhance erythropoiesis when administered to anemic adult subjects . Agents that activate MST1r (*e.g.*, such as MSP and/or MST1r binding fragments thereof) can inhibit erythropoiesis when administered to subjects with erythrocytosis (*e.g.*, subjects with polycythemia vera). Exemplary MST1r nucleic acid and amino acid sequences are provided in Figure 8 (SEQ ID NOs: 1 and 2, respectively). Exemplary MSP nucleic acid and amino acid sequences are provided in Figure 9 (SEQ ID NOs: 3 and 4, respectively).

RYK belongs to the receptor tyrosine kinase (RTK) family but has an inactive kinase domain and functions in non-canonical Wnt signal transduction. As disclosed herein, engagement of RYK by its ligand WNT5a inhibits erythropoiesis. Thus, agents that activate RYK (*e.g.*, such as WNT5a and/or RYK binding fragments thereof) can inhibit erythropoiesis when administered to subjects with erythrocytosis (*e.g.*, subjects with polycythemia vera). Agents that inhibit the interaction between RYK and WNT5a (*e.g.*, an agent that directly disrupts the interaction between RYK and WNT5a, such as an RYK-specific or WNT5a-specific antibody and/or an agent that inhibits RYK and/or WNT5a expression, such as a RYK-specific and/or WNT5a-specific inhibitory nucleic acid) can enhance erythropoiesis when administered to anemic subjects. Exemplary RYK nucleic acid and amino acid sequences are provided in Figure 10 (SEQ ID NOs: 5 and 6, respectively). Exemplary WNT5a nucleic acid and amino acid sequences are provided in Figure 11 (SEQ ID NOs: 7 and 8, respectively).

IL-17ra is one of several receptors for the IL-17 proinflammatory cytokines, which have been reported to be broadly inhibitory on certain bone-marrow progenitors, though the expression of an IL-17 receptor on erythroid progenitors had not been previously documented. As disclosed herein, engagement of IL-17ra by its ligand IL-17a enhances erythropoiesis. Thus, agents that activate IL-17ra (*e.g.*, such as IL-17ra agonists, IL-17a, IL-17f, heterodimeric IL-17a/f ligands and/or IL-17ra binding fragments and/or variants

thereof) can enhance erythropoiesis when administered to anemic subjects. Agents that inhibit the interaction between IL-17ra and IL-17a (*e.g.*, an agent that directly disrupts the interaction between IL-17ra and IL-17a, such as an IL-17ra-specific or IL-17a-specific antibody and/or an agent that inhibits IL-17ra and/or IL-17a expression, such as a IL-17ra-specific and/or IL-17a-specific inhibitory nucleic acid, or an antagonist of IL-17a) can inhibit erythropoiesis when administered to subjects with erythrocytosis (*e.g.*, subjects with polycythemia vera). Exemplary IL-17ra nucleic acid and amino acid sequences are provided in Figure 12 (SEQ ID NOs: 9 and 10, respectively). Exemplary IL-17a nucleic acid and amino acid sequences are provided in Figure 13 (SEQ ID NOs: 11 and 12, respectively).

In certain embodiments, provided herein are ligand-binding polypeptide fragments of a receptor. Such polypeptides can be useful, for example, for inhibiting the interaction between the respective ligand and receptor as well as for identifying agents that bind to the receptor at the same epitope as the ligand. In some embodiments, provided herein are receptor-binding polypeptide fragments of a ligand described herein. Such polypeptides can be useful, for example, for activating the receptor or for inhibiting the interaction between the respective ligand and receptor as well as for identifying agents that bind to the ligand at the same epitope as the receptor.

In some embodiments, the polypeptides and proteins described herein can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, polypeptides and proteins described herein are produced by recombinant DNA techniques. Alternatively, polypeptides described herein can be chemically synthesized using standard peptide synthesis techniques.

In some embodiments, polypeptides and proteins described herein comprise an amino acid sequence substantially identical SEQ ID NO: 2, 4, 6, 8, 10 or 12. Accordingly, in another embodiment, the polypeptides and proteins described herein comprise an amino acid sequence at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or more identical to SEQ ID NO: 2, 4, 6, 8, 10 or 12.

In certain embodiments, the polypeptides and proteins described herein comprise an amino acid identical to SEQ ID NO: 2, 4, 6, 8, 10 or 12 except for 1 or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) conservative sequence modifications. As used herein, the term "conservative sequence modifications" is intended to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the

amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues of the polypeptides described herein can be replaced with other amino acid residues from the same side chain family and the altered antibody can be tested for retained function using the functional assays described herein.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-identical sequences can be disregarded for comparison purposes). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

In some embodiments, provided herein are chimeric or fusion proteins. As used herein, a "chimeric protein" or "fusion protein" comprises a polypeptide or protein described herein (*e.g.*, those comprising SEQ ID NO: 2, 4, 6, 8, 10 or 12) linked to a distinct polypeptide to which it is not linked in nature. For example, the distinct polypeptide can be fused to the N-terminus or C-terminus of the polypeptide either directly, through a peptide bond, or indirectly through a chemical linker. In some embodiments, the peptide described

herein is linked to an immunoglobulin constant domain (*e.g.*, an IgG constant domain, such as a human IgG constant domain).

A chimeric or fusion polypeptide described herein can be produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety.

The polypeptides and proteins described herein can be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding a polypeptide(s) described herein. Alternatively, such peptides can be synthesized by chemical methods. Methods for expression of heterologous polypeptides in recombinant hosts, chemical synthesis of polypeptides, and *in vitro* translation are well known in the art and are described further in Maniatis et al., Molecular Cloning: A Laboratory Manual (1989), 2nd Ed., Cold Spring Harbor, N. Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, Calif.; Merrifield, J. (1969) J. Am. Chem. Soc. 91:501; Chaiken I. M. (1981) CRC Crit. Rev. Biochem. 11:255; Kaiser et al. (1989) Science 243:187; Merrifield, B. (1986) Science 232:342; Kent, S. B. H. (1988) Annu. Rev. Biochem. 57:957; and Offord, R. E. (1980) Semisynthetic Proteins, Wiley Publishing, which are incorporated herein by reference.

Polypeptide Agents

In certain embodiments, a polypeptide agent is used in the methods and compositions disclosed herein. In some embodiments, the polypeptide agent is an isolated polypeptide comprising a erythropoiesis modulating protein described herein or fraction thereof required to bind to and stimulate its corresponding receptor. In some embodiments, the polypeptide

agent comprises an amino acid sequence substantially identical SEQ ID NO: 4, 8 or 12. Accordingly, in another embodiment, the polypeptides agent comprises an amino acid sequence at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or more identical to SEQ ID NO: 4, 8 or 12. In some embodiments, the polypeptide comprises at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90 or 100 consecutive amino acids of SEQ ID NO: 4, 8 or 12.

In some embodiments, the polypeptide agent comprises an amino acid sequence at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or more identical to the amino acid sequence of a IL-17ra ligand.

In some embodiments, the polypeptide agents disclosed herein can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, polypeptide agents are produced by recombinant DNA techniques. Alternatively, polypeptides disclosed herein can be chemically synthesized using standard peptide synthesis techniques.

In certain embodiments, the polypeptide agents described herein comprise an amino acid identical to SEQ ID NO: 4, 8 or 12 except for 1 or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) conservative sequence modifications. As used herein, the term "conservative sequence modifications" is intended to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues of the polypeptides described herein can be

replaced with other amino acid residues from the same side chain family and the altered antibody can be tested for retained function using the functional assays described herein.

In some embodiments, the polypeptide agent is a chimeric or fusion proteins. As used herein, a "chimeric protein" or "fusion protein" comprises a polypeptide or protein described herein (*e.g.*, those comprising SEQ ID NO: 4, 8 or 12 or a fragment thereof) linked to a distinct polypeptide to which it is not linked in nature. For example, the distinct polypeptide can be fused to the N-terminus or C-terminus of the polypeptide either directly, through a peptide bond, or indirectly through a chemical linker. In some embodiments, the peptide described herein is linked to an immunoglobulin constant domain (*e.g.*, an IgG constant domain, such as a human IgG constant domain).

The polypeptide agents provided herein can be generated according to any method available in the art. For example, the polypeptide agents can be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding a polypeptide(s) described herein. Alternatively, such peptides can be synthesized by chemical methods. Methods for expression of heterologous polypeptides in recombinant hosts, chemical synthesis of polypeptides, and *in vitro* translation are well known in the art and are described further in Maniatis et al., Molecular Cloning: A Laboratory Manual (1989), 2nd Ed., Cold Spring Harbor, N. Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, Calif.; Merrifield, J. (1969) J. Am. Chem. Soc. 91:501; Chaiken I. M. (1981) CRC Crit. Rev. Biochem. 11:255; Kaiser et al. (1989) Science 243:187; Merrifield, B. (1986) Science 232:342; Kent, S. B. H. (1988) Annu. Rev. Biochem. 57:957; and Offord, R. E. (1980) Semisynthetic Proteins, Wiley Publishing, which are incorporated herein by reference.

Antibody Agents

In certain embodiments, the methods and compositions provided herein relate to antibodies and antigen binding fragments thereof that bind specifically to an erythropoiesis modulating protein described herein. In some embodiments, the antibodies inhibit the interaction between a receptor provided herein and its corresponding ligand. In some embodiments, the antibodies bind to and activate the receptor provided herein. Such antibodies can be polyclonal or monoclonal and can be, for example, murine, chimeric, humanized or fully human.

Polyclonal antibodies can be prepared by immunizing a suitable subject (*e.g.* a mouse) with a polypeptide antigen (*e.g.*, a polypeptide having a sequence of SEQ ID NOs: 2, 4, 6, 8, 10 or 12 or a fragment thereof). The polypeptide antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized polypeptide. If desired, the antibody directed against the antigen can be isolated from the mammal (*e.g.*, from the blood) and further purified by well-known techniques, such as protein A chromatography to obtain the IgG fraction.

At an appropriate time after immunization, *e.g.*, when the antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies using standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also Brown *et al.* (1981) *J. Immunol.* 127:539-46; Brown *et al.* (1980) *J. Biol. Chem.* 255:4980-83; Yeh *et al.* (1976) *Proc. Natl. Acad. Sci.* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol. Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally Kenneth, R. H. in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); Lerner, E. A. (1981) *Yale J. Biol. Med.* 54:387-402; Gefter, M. L. *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with an immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds to the polypeptide antigen, preferably specifically.

As an alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal specific for a receptor or ligand provided herein can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library or an antibody yeast display library) with the appropriate polypeptide (*e.g.* a polypeptide having a sequence of SEQ ID NOs: 2, 4, 6, 8, 10 or 12 or a fragment thereof) to thereby isolate immunoglobulin library members that bind the polypeptide.

Additionally, recombinant antibodies specific for a receptor or ligand provided herein, such as chimeric or humanized monoclonal antibodies, can be made using standard recombinant DNA techniques. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in US Pat No. 4,816,567; US Pat. No. 5,565,332; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci.* 84:214-218; Nishimura *et al.* (1987) *Cancer Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *Biotechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

Human monoclonal antibodies specific for a receptor or ligand provided herein can be generated using transgenic or transchromosomal mice carrying parts of the human immune system rather than the mouse system. For example, "HuMAb mice" which contain a human immunoglobulin gene miniloci that encodes unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (Lonberg, N. *et al.* (1994) Nature 368(6474): 856 859). Accordingly, the mice exhibit reduced expression of mouse IgM or κ, and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgGκ monoclonal antibodies (Lonberg, N. *et al.* (1994), supra; reviewed in Lonberg, N. (1994) Handbook of Experimental Pharmacology 113:49 101; Lonberg, N. and Huszar, D. (1995) Intern. Rev. Immunol. Vol. 13: 65 93, and Harding, F. and Lonberg, N. (1995) Ann. N. Y Acad. Sci 764:536 546). The preparation of HuMAb mice is described in Taylor, L. *et al.* (1992) Nucleic Acids Research 20:6287 6295; Chen, J. *et al.* (1993) International Immunology 5: 647 656; Tuaillon *et al.* (1993) Proc. Natl. Acad. Sci USA 90:3720 3724; Choi *et al.* (1993) Nature Genetics 4:117 123; Chen, J. *et al.* (1993) EMBO J. 12: 821 830; Tuaillon *et al.* (1994) J. Immunol. 152:2912 2920; Lonberg *et al.*, (1994) Nature 368(6474): 856 859; Lonberg, N. (1994) Handbook of Experimental Pharmacology 113:49 101; Taylor, L. *et al.* (1994) International Immunology 6: 579 591; Lonberg, N. and Huszar, D. (1995) Intern. Rev. Immunol. Vol. 13: 65 93; Harding, F. and Lonberg, N. (1995) Ann. N.Y. Acad. Sci

764:536 546; Fishwild, D. *et al.* (1996) Nature Biotechnology 14: 845 851. See further, U.S. Pat. Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; 5,770,429; and 5,545,807.

In certain embodiments, the antibodies provided herein are able to bind to a receptor or ligand described herein with a dissociation constant of no greater than 10^{-6}, 10^{-7}, 10^{-8} or 10^{-9} M. Standard assays to evaluate the binding ability of the antibodies are known in the art, including for example, ELISAs, Western blots and RIAs. The binding kinetics (*e.g.*, binding affinity) of the antibodies also can be assessed by standard assays known in the art, such as by Biacore analysis. In some embodiments, the binding of the antibody to a receptor described herein substantially inhibits the ability of the corresponding ligand to bind to the receptor. In some embodiments, the binding of the antibody to a ligand described herein substantially inhibits the ability of the ligand to bind to the corresponding receptor. As used herein, an antibody substantially inhibits binding of a receptor and a ligand when an excess of polypeptide reduces the quantity of receptor bound to ligand by at least about 20%, 40%, 60% or 80%, 85% or 90% (as measured in an *in vitro* competitive binding assay).

Small Molecule Agents

Certain embodiments of the methods and compositions disclosed herein relate to methods of enhancing and/or inhibiting erythropoiesis. These methods include administering an agent that inhibits the interaction between a receptor and ligand described herein and/or that stimulates a receptor described herein. Such agents include those disclosed below, those known in the art and those identified using the screening assays described herein. For example, in some embodiments the agent is a RYK inhibitor. Examples of RYK inhibitors include gefitinib (Iressa®), sunitinib (Sutent®; SU11248), erlotinib (Tarceva®; OSI-1774), lapatinib (GW572016; GW2016), canertinib (CI 1033), semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), imatinib (Gleevec®; STI571), dasatinib (BMS-354825), leflunomide (SU101), vandetanib (Zactima™; ZD6474), pharmaceutically acceptable salts thereof.

In some embodiments, assays used to identify agents useful in the methods described herein include a reaction between an receptor polypeptide disclosed herein (*e.g.*, having a sequence of SEQ ID NO: 2, 6 or 10) or a fragment thereof and/or a ligand polypeptide disclosed herein (*e.g.*, having a sequence of SEQ ID NO: 4, 8 or 12) or a fragment thereof

and a test compound. Agents identified *via* such assays, may be useful, for example, for enhancing or inhibiting erythropoiesis.

Agents useful in the methods disclosed herein may be obtained from any available source, including systematic libraries of natural and/or synthetic compounds. Agents may also be obtained by any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, *e.g.,* Zuckermann *et al.*, 1994, *J. Med. Chem.* 37:2678-85); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, 1997, *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of agents may be presented in solution (*e.g.,* Houghten, 1992, *Biotechniques* 13:412-421), or on beads (Lam, 1991, *Nature* 354:82-84), chips (Fodor, 1993, *Nature* 364:555-556), bacteria and/or spores, (Ladner, USP 5,223,409), plasmids (Cull *et al*, 1992, *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith, 1990, *Science* 249:386-390; Devlin, 1990, *Science* 249:404-406; Cwirla *et al,* 1990, *Proc. Natl. Acad. Sci.* 87:6378-6382; Felici, 1991, *J. Mol. Biol.* 222:301-310; Ladner, *supra.*).

Agents useful in the methods disclosed herein may be identified, for example, using assays for screening candidate or test compounds which inhibit complex formation between a receptor provided herein and a ligand described herein.

The basic principle of the assay systems used to identify compounds that inhibit complex formation between a receptor provided herein and a ligand described herein involves preparing a reaction mixture containing a receptor provided herein or fragment

thereof and a ligand described herein or fragment thereof under conditions and for a time sufficient to allow the receptor provided herein or fragment thereof to form a complex with the ligand described herein or fragment thereof. In order to test an agent for modulatory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound can be initially included in the reaction mixture, or can be added at a time subsequent to the addition of the ligand described herein or fragment thereof and the receptor provided herein or fragment thereof. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the ligand described herein or fragment thereof and the receptor provided herein or fragment thereof is then detected. The formation of a complex in the control reaction, but less or no such formation in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the ligand described herein or fragment thereof and the receptor provided herein or fragment thereof.

The assay for compounds that modulate the interaction of the ligand described herein or fragment thereof and the receptor provided herein or fragment thereof may be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the ligand described herein or fragment thereof or the receptor provided herein or fragment thereof onto a solid phase and detecting complexes anchored to the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the ligand described herein or fragment thereof and the receptor provided herein or fragment thereof (*e.g.*, by competition) can be identified by conducting the reaction in the presence of the test substance, *i.e.*, by adding the test substance to the reaction mixture prior to or simultaneously with the ligand described herein or fragment thereof and the receptor provided herein or fragment thereof. Alternatively, test compounds that disrupt preformed complexes, *e.g.*, compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are briefly described below.

In a heterogeneous assay system, either the ligand described herein or fragment thereof or the receptor provided herein or fragment thereof is anchored onto a solid surface

or matrix, while the other corresponding non-anchored component may be labeled, either directly or indirectly. In practice, microtitre plates are often utilized for this approach. The anchored species can be immobilized by a number of methods, either non-covalent or covalent, that are typically well known to one who practices the art. Non-covalent attachment can often be accomplished simply by coating the solid surface with a solution of the ligand described herein or fragment thereof or the receptor provided herein or fragment thereof and drying. Alternatively, an immobilized antibody specific for the assay component to be anchored can be used for this purpose.

In related assays, a fusion protein can be provided which adds a domain that allows one or both of the assay components to be anchored to a matrix. For example, glutathione-S-transferase/marker fusion proteins or glutathione-S-transferase/binding partner can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and either the non-adsorbed the ligand described herein or fragment thereof or the receptor provided herein or fragment thereof, and the mixture incubated under conditions conducive to complex formation (*e.g.*, physiological conditions). Following incubation, the beads or microtiter plate wells are washed to remove any unbound assay components, the immobilized complex assessed either directly or indirectly, for example, as described above.

A homogeneous assay may also be used to identify inhibitors of complex formation. This is typically a reaction, analogous to those mentioned above, which is conducted in a liquid phase in the presence or absence of the test compound. The formed complexes are then separated from unreacted components, and the amount of complex formed is determined. As mentioned for heterogeneous assay systems, the order of addition of reactants to the liquid phase can yield information about which test compounds modulate (inhibit or enhance) complex formation and which disrupt preformed complexes.

In such a homogeneous assay, the reaction products may be separated from unreacted assay components by any of a number of standard techniques, including but not limited to: differential centrifugation, chromatography, electrophoresis and immunoprecipitation. In differential centrifugation, complexes of molecules may be separated from uncomplexed molecules through a series of centrifugal steps, due to the different sedimentation equilibria of complexes based on their different sizes and densities (see, for example, Rivas, G., and Minton, A.P., *Trends Biochem Sci* 1993 Aug;18(8):284-7). Standard chromatographic

techniques may also be utilized to separate complexed molecules from uncomplexed ones. For example, gel filtration chromatography separates molecules based on size, and through the utilization of an appropriate gel filtration resin in a column format, for example, the relatively larger complex may be separated from the relatively smaller uncomplexed components. Similarly, the relatively different charge properties of the complex as compared to the uncomplexed molecules may be exploited to differentially separate the complex from the remaining individual reactants, for example through the use of ion-exchange chromatography resins. Such resins and chromatographic techniques are well known to one skilled in the art (see, *e.g.*, Heegaard, 1998, *J Mol. Recognit.* 11:141-148; Hage and Tweed, 1997, *J. Chromatogr. B. Biomed. Sci. Appl.*, 699:499-525). Gel electrophoresis may also be employed to separate complexed molecules from unbound species (see, *e.g.*, Ausubel *et al* (eds.), In: *Current Protocols in Molecular Biology*, J. Wiley & Sons, New York. 1999). In this technique, protein or nucleic acid complexes are separated based on size or charge, for example. In order to maintain the binding interaction during the electrophoretic process, nondenaturing gels in the absence of reducing agent are typically preferred, but conditions appropriate to the particular interactants will be well known to one skilled in the art. Immunoprecipitation is another common technique utilized for the isolation of a protein-protein complex from solution (see, *e.g.*, Ausubel *et al* (eds.), In: *Current Protocols in Molecular Biology*, J. Wiley & Sons, New York. 1999). In this technique, all proteins binding to an antibody specific to one of the binding molecules are precipitated from solution by conjugating the antibody to a polymer bead that may be readily collected by centrifugation. The bound assay components are released from the beads (through a specific proteolysis event or other technique well known in the art which will not disturb the protein-protein interaction in the complex), and a second immunoprecipitation step is performed, this time utilizing antibodies specific for the correspondingly different interacting assay component. In this manner, only formed complexes should remain attached to the beads. Variations in complex formation in both the presence and the absence of a test compound can be compared, thus offering information about the ability of the compound to modulate interactions between the ligand described herein or fragment thereof and the receptor provided herein or fragment thereof.

Interfering Nucleic Acid Agents

In certain embodiments, interfering nucleic acid molecules that selectively target a receptor provided herein or a ligand described herein are provided herein and/or used in methods described herein. Interfering nucleic acids generally include a sequence of cyclic subunits, each bearing a base-pairing moiety, linked by intersubunit linkages that allow the base-pairing moieties to hybridize to a target sequence in a nucleic acid (typically an RNA) by Watson-Crick base pairing, to form a nucleic acid:oligomer heteroduplex within the target sequence. Interfering RNA molecules include, but are not limited to, antisense molecules, siRNA molecules, single-stranded siRNA molecules, miRNA molecules and shRNA molecules.

Typically at least 17, 18, 19, 20, 21, 22 or 23 nucleotides of the complement of the target mRNA sequence are sufficient to mediate inhibition of a target transcript. Perfect complementarity is not necessary. In some embodiments, the interfering nucleic acid molecule is double-stranded RNA. The double-stranded RNA molecule may have a 2 nucleotide 3' overhang. In some embodiments, the two RNA strands are connected via a hairpin structure, forming a shRNA molecule. shRNA molecules can contain hairpins derived from microRNA molecules. For example, an RNAi vector can be constructed by cloning the interfering RNA sequence into a pCAG-miR30 construct containing the hairpin from the miR30 miRNA. RNA interference molecules may include DNA residues, as well as RNA residues.

Interfering nucleic acid molecules provided herein can contain RNA bases, non-RNA bases or a mixture of RNA bases and non-RNA bases. For example, interfering nucleic acid molecules provided herein can be primarily composed of RNA bases but also contain DNA bases or non-naturally occurring nucleotides.

The interfering nucleic acids can employ a variety of oligonucleotide chemistries. Examples of oligonucleotide chemistries include, without limitation, peptide nucleic acid (PNA), linked nucleic acid (LNA), phosphorothioate, 2'O-Me-modified oligonucleotides, and morpholino chemistries, including combinations of any of the foregoing. In general, PNA and LNA chemistries can utilize shorter targeting sequences because of their relatively high target binding strength relative to 2'O-Me oligonucleotides. Phosphorothioate and 2'O-Me-modified chemistries are often combined to generate 2'O-Me-modified oligonucleotides

having a phosphorothioate backbone. See, *e.g.*, PCT Publication Nos. WO/2013/112053 and WO/2009/008725, incorporated by reference in their entireties.

Peptide nucleic acids (PNAs) are analogs of DNA in which the backbone is structurally homomorphous with a deoxyribose backbone, consisting of N-(2-aminoethyl) glycine units to which pyrimidine or purine bases are attached. PNAs containing natural pyrimidine and purine bases hybridize to complementary oligonucleotides obeying Watson-Crick base-pairing rules, and mimic DNA in terms of base pair recognition (Egholm, Buchardt et al. 1993). The backbone of PNAs is formed by peptide bonds rather than phosphodiester bonds, making them well-suited for antisense applications (see structure below). The backbone is uncharged, resulting in PNA/DNA or PNA/RNA duplexes that exhibit greater than normal thermal stability. PNAs are not recognized by nucleases or proteases.

Despite a radical structural change to the natural structure, PNAs are capable of sequence-specific binding in a helix form to DNA or RNA. Characteristics of PNAs include a high binding affinity to complementary DNA or RNA, a destabilizing effect caused by single-base mismatch, resistance to nucleases and proteases, hybridization with DNA or RNA independent of salt concentration and triplex formation with homopurine DNA. PANAGENE.TM. has developed its proprietary Bts PNA monomers (Bts; benzothiazole-2-sulfonyl group) and proprietary oligomerization process. The PNA oligomerization using Bts PNA monomers is composed of repetitive cycles of deprotection, coupling and capping. PNAs can be produced synthetically using any technique known in the art. See, *e.g.*, U.S. Pat. Nos. 6,969,766, 7,211,668, 7,022,851, 7,125,994, 7,145,006 and 7,179,896. See also U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262 for the preparation of PNAs. Further teaching of PNA compounds can be found in Nielsen et al., Science, 254:1497-1500, 1991. Each of the foregoing is incorporated by reference in its entirety.

Interfering nucleic acids may also contain "locked nucleic acid" subunits (LNAs). "LNAs" are a member of a class of modifications called bridged nucleic acid (BNA). BNA is characterized by a covalent linkage that locks the conformation of the ribose ring in a C30-endo (northern) sugar pucker. For LNA, the bridge is composed of a methylene between the 2'-O and the 4'-C positions. LNA enhances backbone preorganization and base stacking to increase hybridization and thermal stability.

The structures of LNAs can be found, for example, in Wengel, et al., Chemical Communications (1998) 455; Tetrahedron (1998) 54:3607, and Accounts of Chem. Research (1999) 32:301); Obika, et al., Tetrahedron Letters (1997) 38:8735; (1998) 39:5401, and Bioorganic Medicinal Chemistry (2008) 16:9230. Compounds provided herein may incorporate one or more LNAs; in some cases, the compounds may be entirely composed of LNAs. Methods for the synthesis of individual LNA nucleoside subunits and their incorporation into oligonucleotides are described, for example, in U.S. Pat. Nos. 7,572,582, 7,569,575, 7,084,125, 7,060,809, 7,053,207, 7,034,133, 6,794,499, and 6,670,461, each of which is incorporated by reference in its entirety. Typical intersubunit linkers include phosphodiester and phosphorothioate moieties; alternatively, non-phosphorous containing linkers may be employed. One embodiment is an LNA containing compound where each LNA subunit is separated by a DNA subunit. Certain compounds are composed of alternating LNA and DNA subunits where the intersubunit linker is phosphorothioate.

"Phosphorothioates" (or S-oligos) are a variant of normal DNA in which one of the nonbridging oxygens is replaced by a sulfur. The sulfurization of the internucleotide bond reduces the action of endo-and exonucleases including 5' to 3' and 3' to 5' DNA POL 1 exonuclease, nucleases S1 and P1, RNases, serum nucleases and snake venom phosphodiesterase. Phosphorothioates are made by two principal routes: by the action of a solution of elemental sulfur in carbon disulfide on a hydrogen phosphonate, or by the method of sulfurizing phosphite triesters with either tetraethylthiuram disulfide (TETD) or 3H-1, 2-bensodithiol-3-one 1, 1-dioxide (BDTD) (see, *e.g.*, Iyer et al., J. Org. Chem. 55, 4693-4699, 1990). The latter methods avoid the problem of elemental sulfur's insolubility in most organic solvents and the toxicity of carbon disulfide. The TETD and BDTD methods also yield higher purity phosphorothioates.

"2'O-Me oligonucleotides" molecules carry a methyl group at the 2'-OH residue of the ribose molecule. 2'-O-Me-RNAs show the same (or similar) behavior as DNA, but are protected against nuclease degradation. 2'-O-Me-RNAs can also be combined with phosphothioate oligonucleotides (PTOs) for further stabilization. 2'O-Me oligonucleotides (phosphodiester or phosphothioate) can be synthesized according to routine techniques in the art (see, *e.g.*, Yoo et al., Nucleic Acids Res. 32:2008-16, 2004).

The interfering nucleic acids described herein may be contacted with a cell or administered to an organism (*e.g.*, a human). Alternatively, constructs and/or vectors

encoding the interfering RNA molecules may be contacted with or introduced into a cell or organism. In certain embodiments, a viral, retroviral or lentiviral vector is used. In some embodiments, the vector has a tropism for cardiac tissue. In some embodiments the vector is an adeno-associated virus.

Typically at least 17, 18, 19, 20, 21, 22 or 23 nucleotides of the complement of the target mRNA sequence are sufficient to mediate inhibition of a target transcript. Perfect complementarity is not necessary. In some embodiments, the interfering nucleic acids contains a 1, 2 or 3 nucleotide mismatch with the target sequence. The interfering nucleic acid molecule may have a 2 nucleotide 3' overhang. If the interfering nucleic acid molecule is expressed in a cell from a construct, for example from a hairpin molecule or from an inverted repeat of the desired sequence, then the endogenous cellular machinery will create the overhangs. shRNA molecules can contain hairpins derived from microRNA molecules. For example, an RNAi vector can be constructed by cloning the interfering RNA sequence into a pCAG-miR30 construct containing the hairpin from the miR30 miRNA. RNA interference molecules may include DNA residues, as well as RNA residues.

In some embodiments, the interfering nucleic acid molecule is a siRNA molecule. Such siRNA molecules should include a region of sufficient homology to the target region, and be of sufficient length in terms of nucleotides, such that the siRNA molecule down-regulate target RNA. The term "ribonucleotide" or "nucleotide" can, in the case of a modified RNA or nucleotide surrogate, also refer to a modified nucleotide, or surrogate replacement moiety at one or more positions. It is not necessary that there be perfect complementarity between the siRNA molecule and the target, but the correspondence must be sufficient to enable the siRNA molecule to direct sequence-specific silencing, such as by RNAi cleavage of the target RNA. In some embodiments, the sense strand need only be sufficiently complementary with the antisense strand to maintain the overall double-strand character of the molecule.

In addition, an siRNA molecule may be modified or include nucleoside surrogates. Single stranded regions of an siRNA molecule may be modified or include nucleoside surrogates, *e.g.*, the unpaired region or regions of a hairpin structure, *e.g.*, a region which links two complementary regions, can have modifications or nucleoside surrogates. Modification to stabilize one or more 3′- or 5′-terminus of an siRNA molecule, *e.g.*, against exonucleases, or to favor the antisense siRNA agent to enter into RISC are also useful.

Modifications can include C3 (or C6, C7, C12) amino linkers, thiol linkers, carboxyl linkers, non-nucleotidic spacers (C3, C6, C9, C12, abasic, triethylene glycol, hexaethylene glycol), special biotin or fluorescein reagents that come as phosphoramidites and that have another DMT-protected hydroxyl group, allowing multiple couplings during RNA synthesis.

Each strand of an siRNA molecule can be equal to or less than 35, 30, 25, 24, 23, 22, 21, or 20 nucleotides in length. In some embodiments, the strand is at least 19 nucleotides in length. For example, each strand can be between 21 and 25 nucleotides in length. In some embodiments, siRNA agents have a duplex region of 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs, and one or more overhangs, such as one or two 3′ overhangs, of 2-3 nucleotides.

A "small hairpin RNA" or "short hairpin RNA" or "shRNA" includes a short RNA sequence that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. The shRNAs provided herein may be chemically synthesized or transcribed from a transcriptional cassette in a DNA plasmid. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RNA-induced silencing complex (RISC).

In some embodiments, shRNAs are about 15-60, 15-50, or 15-40 (duplex) nucleotides in length, about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, or are about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.*, each complementary sequence of the double-stranded shRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, or about 20-24, 21-22, or 21-23 nucleotides in length, and the double-stranded shRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, or about 18-22, 19-20, or 19-21 base pairs in length). shRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides on the antisense strand and/or 5'-phosphate termini on the sense strand. In some embodiments, the shRNA comprises a sense strand and/or antisense strand sequence of from about 15 to about 60 nucleotides in length (*e.g.*, about 15-60, 15-55, 15-50, 15-45, 15-40, 15-35, 15-30, or 15-25 nucleotides in length),or from about 19 to about 40 nucleotides in length (*e.g.*, about 19-40, 19-35, 19-30, or 19-25 nucleotides in length), or from about 19 to about 23 nucleotides in length (*e.g.*, 19, 20, 21, 22, or 23 nucleotides in length).

Non-limiting examples of shRNA include a double-stranded polynucleotide molecule assembled from a single-stranded molecule, where the sense and antisense regions are linked

by a nucleic acid-based or non-nucleic acid-based linker; and a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions. In some embodiments, the sense and antisense strands of the shRNA are linked by a loop structure comprising from about 1 to about 25 nucleotides, from about 2 to about 20 nucleotides, from about 4 to about 15 nucleotides, from about 5 to about 12 nucleotides, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more nucleotides.

Additional embodiments related to the shRNAs, as well as methods of designing and synthesizing such shRNAs, are described in U.S. patent application publication number 2011/0071208, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In some embodiments, provided herein are micro RNAs (miRNAs). miRNAs represent a large group of small RNAs produced naturally in organisms, some of which regulate the expression of target genes. miRNAs are formed from an approximately 70 nucleotide single-stranded hairpin precursor transcript by Dicer. miRNAs are not translated into proteins, but instead bind to specific messenger RNAs, thereby blocking translation. In some instances, miRNAs base-pair imprecisely with their targets to inhibit translation.

In some embodiments, antisense oligonucleotide compounds are provided herein. In certain embodiments, the degree of complementarity between the target sequence and antisense targeting sequence is sufficient to form a stable duplex. The region of complementarity of the antisense oligonucleotides with the target RNA sequence may be as short as 8-11 bases, but can be 12-15 bases or more, *e.g.*, 10-40 bases, 12-30 bases, 12-25 bases, 15-25 bases, 12-20 bases, or 15-20 bases, including all integers in between these ranges. An antisense oligonucleotide of about 14-15 bases is generally long enough to have a unique complementary sequence.

In certain embodiments, antisense oligonucleotides may be 100% complementary to the target sequence, or may include mismatches, *e.g.*, to improve selective targeting of allele containing the disease-associated mutation, as long as a heteroduplex formed between the oligonucleotide and target sequence is sufficiently stable to withstand the action of cellular nucleases and other modes of degradation which may occur *in vivo*. Hence, certain oligonucleotides may have about or at least about 70% sequence complementarity, *e.g.*, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%,

87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence complementarity, between the oligonucleotide and the target sequence. Oligonucleotide backbones that are less susceptible to cleavage by nucleases are discussed herein. Mismatches, if present, are typically less destabilizing toward the end regions of the hybrid duplex than in the middle. The number of mismatches allowed will depend on the length of the oligonucleotide, the percentage of G:C base pairs in the duplex, and the position of the mismatch(es) in the duplex, according to well understood principles of duplex stability.

Interfering nucleic acid molecules can be prepared, for example, by chemical synthesis, in vitro transcription, or digestion of long dsRNA by Rnase III or Dicer. These can be introduced into cells by transfection, electroporation, or other methods known in the art. See Hannon, GJ, 2002, RNA Interference, Nature 418: 244-251; Bernstein E et al., 2002, The rest is silence. RNA 7: 1509-1521; Hutvagner G et al., RNAi: Nature abhors a double-strand. Curr. Opin. Genetics & Development 12: 225-232; Brummelkamp, 2002, A system for stable expression of short interfering RNAs in mammalian cells. Science 296: 550-553; Lee NS, Dohjima T, Bauer G, Li H, Li M-J, Ehsani A, Salvaterra P, and Rossi J. (2002). Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nature Biotechnol. 20:500-505; Miyagishi M, and Taira K. (2002). U6-promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. Nature Biotechnol. 20:497-500; Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, and Conklin DS. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes & Dev. 16:948-958; Paul CP, Good PD, Winer I, and Engelke DR. (2002). Effective expression of small interfering RNA in human cells. Nature Biotechnol. 20:505-508; Sui G, Soohoo C, Affar E-B, Gay F, Shi Y, Forrester WC, and Shi Y. (2002). A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. USA 99(6):5515-5520; Yu J-Y, DeRuiter SL, and Turner DL. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc. Natl. Acad. Sci. USA 99(9):6047-6052.

In the present methods, an interfering nucleic acid molecule or an interfering nucleic acid encoding polynucleotide can be administered to the subject, for example, as naked nucleic acid, in combination with a delivery reagent, and/or as a nucleic acid comprising sequences that express an interfering nucleic acid molecule. In some embodiments the

nucleic acid comprising sequences that express the interfering nucleic acid molecules are delivered within vectors, *e.g.* plasmid, viral and bacterial vectors. Any nucleic acid delivery method known in the art can be used in the methods described herein. Suitable delivery reagents include, but are not limited to, *e.g.*, the Mirus Transit TKO lipophilic reagent; lipofectin; lipofectamine; cellfectin; polycations (*e.g.*, polylysine), atelocollagen, nanoplexes and liposomes. The use of atelocollagen as a delivery vehicle for nucleic acid molecules is described in Minakuchi *et al.* Nucleic Acids Res., 32(13):e109 (2004); Hanai *et al.* Ann NY Acad Sci., 1082:9-17 (2006); and Kawata *et al.* Mol Cancer Ther., 7(9):2904-12 (2008); each of which is incorporated herein in their entirety. Exemplary interfering nucleic acid delivery systems are provided in U.S. Patent Nos. 8,283,461, 8,313,772, 8,501,930. 8,426,554, 8,268,798 and 8,324,366, each of which is hereby incorporated by reference in its entirety.

In some embodiments of the methods described herein, liposomes are used to deliver an inhibitory oligonucleotide to a subject. Liposomes suitable for use in the methods described herein can be formed from standard vesicle-forming lipids, which generally include neutral or negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of factors such as the desired liposome size and half-life of the liposomes in the blood stream. A variety of methods are known for preparing liposomes, for example, as described in Szoka *et al.* (1980), Ann. Rev. Biophys. Bioeng. 9:467; and U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, the entire disclosures of which are herein incorporated by reference.

The liposomes for use in the present methods can also be modified so as to avoid clearance by the mononuclear macrophage system ("MMS") and reticuloendothelial system ("RES"). Such modified liposomes have opsonization-inhibition moieties on the surface or incorporated into the liposome structure.

Opsonization-inhibiting moieties for use in preparing the liposomes described herein are typically large hydrophilic polymers that are bound to the liposome membrane. As used herein, an opsonization inhibiting moiety is "bound" to a liposome membrane when it is chemically or physically attached to the membrane, *e.g.*, by the intercalation of a lipid-soluble anchor into the membrane itself, or by binding directly to active groups of membrane lipids. These opsonization-inhibiting hydrophilic polymers form a protective surface layer that significantly decreases the uptake of the liposomes by the MMS and RES; *e.g.*, as

described in U.S. Pat. No. 4,920,016, the entire disclosure of which is herein incorporated by reference.

In some embodiments, opsonization inhibiting moieties suitable for modifying liposomes are water-soluble polymers with a number-average molecular weight from about 500 to about 40,000 daltons, or from about 2,000 to about 20,000 daltons. Such polymers include polyethylene glycol (PEG) or polypropylene glycol (PPG) derivatives; *e.g.*, methoxy PEG or PPG, and PEG or PPG stearate; synthetic polymers such as polyacrylamide or poly N-vinyl pyrrolidone; linear, branched, or dendrimeric polyamidoamines; polyacrylic acids; polyalcohols, *e.g.*, polyvinylalcohol and polyxylitol to which carboxylic or amino groups are chemically linked, as well as gangliosides, such as ganglioside GM1. Copolymers of PEG, methoxy PEG, or methoxy PPG, or derivatives thereof, are also suitable. In addition, the opsonization inhibiting polymer can be a block copolymer of PEG and either a polyamino acid, polysaccharide, polyamidoamine, polyethyleneamine, or polynucleotide. The opsonization inhibiting polymers can also be natural polysaccharides containing amino acids or carboxylic acids, *e.g.*, galacturonic acid, glucuronic acid, mannuronic acid, hyaluronic acid, pectic acid, neuraminic acid, alginic acid, carrageenan; aminated polysaccharides or oligosaccharides (linear or branched); or carboxylated polysaccharides or oligosaccharides, *e.g.*, reacted with derivatives of carbonic acids with resultant linking of carboxylic groups. In some embodiments, the opsonization-inhibiting moiety is a PEG, PPG, or derivatives thereof. Liposomes modified with PEG or PEG-derivatives are sometimes called "PEGylated liposomes."

Pharmaceutical Compositions

In certain embodiments, provided herein is a composition, *e.g.*, a pharmaceutical composition, containing at least one agent described herein together with a pharmaceutically acceptable carrier. In one embodiment, the composition includes a combination of multiple (*e.g.*, two or more) agents described herein.

As described in detail below, the pharmaceutical compositions disclosed herein may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, *e.g.*, those targeted for buccal, sublingual, and systemic absorption, boluses, powders, granules, pastes for application to the tongue; or (2) parenteral administration, for example, by subcutaneous, intramuscular, intravenous, intrathecal,

intracerebral or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation.

Methods of preparing these formulations or compositions include the step of bringing into association an agent described herein with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association an agent described herein with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Pharmaceutical compositions suitable for parenteral administration comprise one or more agents described herein in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain sugars, alcohols, antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions include water, ethanol, dimethyl sulfoxide (DMSO), polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

Regardless of the route of administration selected, the agents provided herein, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions disclosed herein, are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art.

Therapeutic Methods

In certain embodiments, provided herein are methods of stimulating erythropoiesis in a subject (*e.g.*, a subject in need thereof, such as a subject with anemia) or inhibiting erythropoiesis in a subject (*e.g.*, a subject in need thereof, such as a subject with erythrocytosis) by administering to the subject an agent or pharmaceutical composition provided herein.

In some embodiments, the methods provided herein relate to the stimulation of erythropoiesis in a subject (*e.g.*, a human subject). In some embodiments, the subject is an infant. In some embodiments, the subject is a subject in need thereof. In some embodiments, the subject in anemic. In some embodiments, the subject has congenital anemia. The subject may have iron deficiency anemia. In some embodiments, the subject has Diamond Blackfan Anemia. In some embodiments, the subject has refractory anemia. The subject may be refractory to erythropoietin. The subject may have myelodysplastic syndrome. The subject may have anemia as a result of myelosuppressive chemotherapy. The subject may have cancer.

In some embodiments, the subject is being treated with erythropoietin or an erythropoietin derivative. In some embodiments, the erythropoietin or erythropoietin derivative is asialoerythropoietin, N-deglycosylated erythropoietin, O-deglycosylated erythropoietin, erythropoietin with reduced carbohydrate content, erythropoietin with altered glycosylation patterns, erythropoietin with carbohydrates oxidized then reduced, arylglyoxal-modified erythropoietin, alkylglyoxal-modified erythropoietin, 2,3-butanedione-modified erythropoietin, cyclohexanedione-modified erythropoietin, biotinylated erythropoietin, N-alkylated-lysyl-erythropoietin, glucitolyl lysine erythropoietin, alpha-deoxy-alpha-fructosyllysine-erythropoietin, carbamylated erythropoietin, acetylated erythropoietin, succinylated erythropoietin, alpha-carboxyalkyl erythropoietin, nitrated erythropoietin, iodinated erythropoietin.

In some embodiments, the subject has a cancer-associated anemia. In some embodiments the cancer-associated anemia is a cancer is such as, but not limited to, breast, prostate, lung, skin, colorectal, cervical, pancreatic, ovarian, lymphoma, leukemia, liver, testicular, and brain cancer.

In some embodiments, provided herein is a method of stimulating erythropoiesis in a subject with anemia comprising administering to the subject an agent that binds to MST1r. In some embodiments the agent induces MST1r activity. In some embodiments, the agent is a polypeptide agent (*e.g.*, a MSP ligand protein or a fragment thereof). In some embodiments, the agent is an antibody agent. In some embodiments, the agent is a small molecule agent. In some embodiments, the method further comprises administering to the subject erythropoietin or an erythropoietin derivative. In some embodiments, the erythropoietin or erythropoietin derivative is asialoerythropoietin, N-deglycosylated erythropoietin, O-deglycosylated

erythropoietin, erythropoietin with reduced carbohydrate content, erythropoietin with altered glycosylation patterns, erythropoietin with carbohydrates oxidized then reduced, arylglyoxal-modified erythropoietin, alkylglyoxal-modified erythropoietin, 2,3-butanedione-modified erythropoietin, cyclohexanedione-modified erythropoietin, biotinylated erythropoietin, N-alkylated-lysyl-erythropoietin, glucitolyl lysine erythropoietin, alpha-deoxy-alpha-fructosyllysine-erythropoietin, carbamylated erythropoietin, acetylated erythropoietin, succinylated erythropoietin, alpha-carboxyalkyl erythropoietin, nitrated erythropoietin, iodinated erythropoietin..

In some embodiments, provided herein is a method of stimulating erythropoiesis in a subject with anemia comprising administering to the subject an agent that binds to IL-17ra. In some embodiments the agent induces IL-17ra activity. In some embodiments, the agent is a polypeptide agent (*e.g.*, an IL-17a protein or a fragment thereof, an IL-17f protein or a fragment thereof, or a IL-17a/f heterodimeric polypeptide). In some embodiments, the agent is an antibody agent. In some embodiments, the agent is a small molecule agent. In some embodiments, the method further comprises administering to the subject erythropoietin or an erythropoietin derivative. In some embodiments, the erythropoietin or erythropoietin derivative is asialoerythropoietin, N-deglycosylated erythropoietin, O-deglycosylated erythropoietin, erythropoietin with reduced carbohydrate content, erythropoietin with altered glycosylation patterns, erythropoietin with carbohydrates oxidized then reduced, arylglyoxal-modified erythropoietin, alkylglyoxal-modified erythropoietin, 2,3-butanedione-modified erythropoietin, cyclohexanedione-modified erythropoietin, biotinylated erythropoietin, N-alkylated-lysyl-erythropoietin, glucitolyl lysine erythropoietin, alpha-deoxy-alpha-fructosyllysine-erythropoietin, carbamylated erythropoietin, acetylated erythropoietin, succinylated erythropoietin, alpha-carboxyalkyl erythropoietin, nitrated erythropoietin, iodinated erythropoietin..

In some embodiments, provided herein is a method of stimulating erythropoiesis in a subject with anemia comprising administering to the subject an agent that inhibits the binding of WNT5a to RYK and/or inhibits WNT5a and/or RYK expression or activity. In some embodiments the agent inhibits RYK activity. In some embodiments, the agent is a polypeptide agent (*e.g.*, non-activating WNT5a or RYK polypeptide or fragment thereof). In some embodiments, the agent is an antibody agent. In some embodiments, the agent is a small molecule agent. In some embodiments, the agent is an inhibitory nucleic acid agent. In

some embodiments, the method further comprises administering to the subject erythropoietin or an erythropoietin derivative. In some embodiments, the erythropoietin or erythropoietin derivative is asialoerythropoietin, N-deglycosylated erythropoietin, O-deglycosylated erythropoietin, erythropoietin with reduced carbohydrate content, erythropoietin with altered glycosylation patterns, erythropoietin with carbohydrates oxidized then reduced, arylglyoxal-modified erythropoietin, alkylglyoxal-modified erythropoietin, 2,3-butanedione-modified erythropoietin, cyclohexanedione-modified erythropoietin, biotinylated erythropoietin, N-alkylated-lysyl-erythropoietin, glucitolyl lysine erythropoietin, alpha-deoxy-alpha-fructosyllysine-erythropoietin, carbamylated erythropoietin, acetylated erythropoietin, succinylated erythropoietin, alpha-carboxyalkyl erythropoietin, nitrated erythropoietin, iodinated erythropoietin..

In some embodiments, the methods provided herein relate to the inhibition of erythropoiesis in a subject (*e.g.*, a human subject). In some embodiments, the subject is a subject in need thereof. In some embodiments, the subject has erythrocytosis (*e.g.*, primary or secondary erythrocytosis). In some embodiments, the subject has polycythemia vera. In some embodiments, the subject is being treated with a Jak2 inhibitor. In some embodiments, the Jak2 inhibitor is ruxolitinib, fedratinib, tofacitinib, baricitinib (INCB039110), lestaurtinib (CEP701), pacritinib (SB1518), XL019, AZD1480, gandotinib (LY2784544), BMS911543, fedratinib (SAR302503), decemotinib (V-509), INCB39110, GEN1, GEN2, GLPG0634, NS018, and N-(cyanomethyl)-4-[2-(4-morpholinoanilino)pyrimidin-4-yl]benzamide; or pharmaceutically acceptable salts thereof.

In some embodiments, provided herein is a method of inhibiting erythropoiesis in a subject with anemia comprising administering to the subject an agent that that inhibits the binding of MST1r to MSP and/or inhibits MST1r and/or MSP expression or activity. In some embodiments the agent inhibits MST1r activity. In some embodiments, the agent is a polypeptide agent (*e.g.*, non-activating MSP or MST1r polypeptide or fragment thereof). In some embodiments, the agent is an antibody agent. In some embodiments, the agent is a small molecule agent. In some embodiments, the agent is an inhibitory nucleic acid agent. In some embodiments, the method further comprises administering to the subject a Jak2 inhibitor. In some embodiments, the Jak2 inhibitor is ruxolitinib, fedratinib, tofacitinib, baricitinib (INCB039110), lestaurtinib (CEP701), pacritinib (SB1518), XL019, AZD1480, gandotinib (LY2784544), BMS911543, fedratinib (SAR302503), decemotinib (V-509),

INCB39110, GEN1, GEN2, GLPG0634, NS018, and N-(cyanomethyl)-4-[2-(4-morpholinoanilino)pyrimidin-4-yl]benzamide; or pharmaceutically acceptable salts thereof.

In some embodiments, provided herein is a method of inhibiting erythropoiesis in a subject with anemia comprising administering to the subject an agent that binds RYK. In some embodiments the agent induces RYK activity. In some embodiments, the agent is a polypeptide agent (*e.g.*, a WNT5a protein or a fragment thereof). In some embodiments, the agent is an antibody agent. In some embodiments, the agent is a small molecule agent. In some embodiments, the agent is an inhibitory nucleic acid agent. In some embodiments, the method further comprises administering to the subject a Jak2 inhibitor. In some embodiments, the Jak2 inhibitor is ruxolitinib, fedratinib, tofacitinib, baricitinib (INCB039110), lestaurtinib (CEP701), pacritinib (SB1518), XL019, AZD1480, gandotinib (LY2784544), BMS911543, fedratinib (SAR302503), decemotinib (V-509), INCB39110, GEN1, GEN2, GLPG0634, NS018, and N-(cyanomethyl)-4-[2-(4-morpholinoanilino)pyrimidin-4-yl]benzamide; or pharmaceutically acceptable salts thereof.

In some embodiments, provided herein is a method of inhibiting erythropoiesis in a subject with anemia comprising administering to the subject an agent that that inhibits the binding of IL-17ra to IL-17a and/or inhibits IL-17ra and/or IL-17a expression or activity. In some embodiments the agent inhibits IL-17ra activity. In some embodiments, the agent is a polypeptide agent (*e.g.*, non-activating IL-17a or IL-17ra polypeptide or fragment thereof). In some embodiments, the agent is an antibody agent. In some embodiments, the agent is a small molecule agent. In some embodiments, the agent is an inhibitory nucleic acid agent. In some embodiments, the method further comprises administering to the subject a Jak2 inhibitor. In some embodiments, the Jak2 inhibitor is ruxolitinib, fedratinib, tofacitinib, baricitinib (INCB039110), lestaurtinib (CEP701), pacritinib (SB1518), XL019, AZD1480, gandotinib (LY2784544), BMS911543, fedratinib (SAR302503), decemotinib (V-509), INCB39110, GEN1, GEN2, GLPG0634, NS018, and N-(cyanomethyl)-4-[2-(4-morpholinoanilino)pyrimidin-4-yl]benzamide; or pharmaceutically acceptable salts thereof.

The pharmaceutical compositions disclosed herein may be delivered by any suitable route of administration, including orally and parenterally. In certain embodiments the pharmaceutical compositions are delivered generally (*e.g.*, via oral or parenteral administration).

In certain aspects, agents and/or compositions disclosed herein may be administered at a dose sufficient to achieve the desired result (*e.g.*, increased or decreased erythropoiesis). In some embodiments, the agent is a ligand of MST1r, RYK, and/or IL-17ra . In some embodiments, the agent is IL-17a or a fragment thereof. In some embodiments, the agent is IL-17f or a fragment thereof. In some embodiments, the agent is heterodimeric IL-17a/f protein or a fragment thereof. In some embodiments, the agent is WNT5 or a fragment thereof. In some embodiments, the agent is MSP or a fragment thereof. The agent may be an agonist of IL-17ra. In some embodiments, the agent is an agent disclosed herein.

In certain embodiments, the method may comprise administering about 1 μg to about 1 gram of agent or composition to the subject, such as about 1 μg to about 1 mg, about 2 μg to about 2 mg, about 3 μg to about 3 mg, about 4 μg to about 4 mg, about 100 μg to about 2 mg, about 200 μg to about 2 mg, about 300 μg to about 3 mg, about 400 μg to about 4 mg, about 250 μg to about 1 mg, or about 250 μg to about 750 μg of the agent or composition. In some embodiments, the method may comprise administering about 25 μg, about 50 μg, about 75 μg, about 100 μg, about 125 μg about 150 μg, about 175 μg about 200 μg, about 225 μg about 250 μg, about 275 μg, about 300 μg, about 325 μg, about 350 μg, about 375 μg, about 400 μg, about 425 μg, about 450 μg, about 475 μg, about 500 μg, about 600 μg, about 650 μg, about 700 μg, about 750 μg, about 800 μg, about 850 μg, about 900 μg, about 950 μg, about 1000 μg, about 1200 μg, about 1250 μg, about 1300 μg, about 1333 μg, about 1350 μg, about 1400 μg, about 1500 μg, about 1667 μg, about 1750 μg, about 1800 μg, about 2000 μg, about 2200 μg, about 2250 μg, about 2300 μg, about 2333 μg, about 2350 μg, about 2400 μg, about 2500 μg, about 2667 μg, about 2750 μg, about 2800 μg, about 3 mg, about 3.3 mg, about 3.5 mg, about 3.7 mg, about 4 mg, about 4.5 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg, or about 10 mg of the agent or composition.

In some embodiments, administering an agent or composition the subject comprises administering a bolus of the composition. The method may comprise administering the composition to the subject at least once per month, twice per month, three times per month. In certain embodiments, the method may comprise administering the composition at least once per week, at least once every two weeks, or once every three weeks. In some embodiments, the method may comprise administering the composition to the subject 1, 2, 3, 4, 5, 6, or 7 times per week.

In some embodiments, the agents and/or compositions described herein may be administered conjointly with erythropoietin or erythropoietin derivative (*e.g.*, an erythropoietin derivative disclosed herein).

In certain embodiments, the compositions of the invention can be administered in a variety of conventional ways. In some aspects, the compositions of the invention are suitable for parenteral administration. In some embodiments, these compositions may be administered, for example, intraperitoneally, intravenously, intrarenally, or intrathecally. In some aspects, the compositions of the invention are injected intravenously.

In some embodiments, actual dosage levels of the active ingredients in the pharmaceutical compositions may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

In general, a suitable daily dose of an agent described herein will be that amount of the agent which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above.

In some embodiments, the methods disclosed herein relate to the use of the growth factors disclosed herein (*e.g.*, IL-17a, WNT5, MSP or IL-17f), in the amplification of human early erythroid progenitors *in vitro*. In recent years, several culture systems have been developed that produce and amplify early erythroid progenitors from CD34+ progenitors, for the purpose of generating transfusable red blood cells *in vitro* (See Migliaccio AR, Whitsett C, Papayannopoulou T, Sadelain M. The potential of stem cells as an *in vitro* source of red blood cells for transfusion can be found in *Cell Stem Cell*. 2012;10(2):115-119, hereby incorporated by reference in its entirety). Details regarding transfusion of *in vitro*-generated red blood cells can be found in *Blood*. 2011;118(19):5071-5079 (hereby incorporated by reference in its entirety). An *in vitro* source of red blood cells may be particularly useful for rare blood types, and for the *in vitro* application of gene therapy for blood genetic diseases (*e.g.* hemoglobinopathies). Sustaining and amplifying early erythroid progenitors in culture is still a challenge. Most culture systems use some combination of gluococorticoids, erythropoietin, stem cell factor and insulin-like growth factor 1 (IGF1) for this purpose. Activating IL-17ra with IL-17a or IL-17f, or inhibiting Ryk and Mst1r with Wnt5a and MSP, respectively, may enhance the amplification and propagation of erythroid progenitors in these systems.

In certain aspects, provided herein are methods of generating red blood cells *in vitro*. In some embodiments, disclosed herein are methods of expanding or propagating progenitor cells *in vitro* comprising supplementing or adding an agent that activates IL-17ra to a cell medium comprising the progenitor cells. In some aspects, disclosed herein are methods of expanding or propagating progenitor cells *in vitro* comprising supplementing or adding an agent that inhibits Ryk and/or Mst1r. The agent many be an agent disclosed herein. In some embodiments, the methods disclosed herein include adding or supplementing *in vitro* medium used for growing or expanding progenitor cells (*e.g.*, CD34+ progenitors or erythroid progenitors) with a composition or agent disclosed herein. In some embodiments, the methods disclosed herein include adding or supplementing cell culture methods used for growing or expanding progenitor cells (*e.g.*, CD34+ progenitors or erythroid progenitors) with a composition or agent disclosed herein. In some embodiments, the methods disclosed herein include adding or supplementing *in vitro* medium used for growing or expanding progenitor cells (*e.g.*, CD34+ progenitors or erythroid progenitors) with a composition or agent disclosed herein.

EXEMPLIFICATION

Example 1: Single-cell RNA-seq of Kit+ hematopoietic progenitors

To define the principal cellular stages of early erythropoiesis, single cell RNA sequencing (scRNA-Seq) were performed on hematopoietic progenitor cells (HPC) isolated from adult mouse bone marrow (BM) at 8 weeks of age, and from the mouse fetal liver (FL) at mid gestation (embryonic day 13.5). BM was isolated in a basal steady state (bBM) from untreated adult mice, or following stimulation with Epo (eBM) for 48 hours to induce an erythropoietic stress response. HPCs were isolated conditional on expression of the cell-surface receptor Kit, expressed on all hematopoietic stem and early progenitor cells (Fig. 1, Panel a). This inclusive approach to cell fractionation preserves the relative abundance of the various early progenitor cell states, allowing unbiased reconstruction of early hematopoietic differentiation.

Across three droplet microfluidic runs, a total of 17,868 cells were isolated, barcoded and sequenced. Following filtering , over 14,000 cells were carried forward for analysis (Fig. 1, Panel b).

To visualize the single cell transcriptomes, a graph-based representation of the data, SPRING, was used where each cell is represented by a single node that is connected by

unweighted edges to its nearest neighboring cells in gene expression space. Cell positions are then projected into two dimensions using a force-directed graph layout (Fig. 1, Panel c). Because the hierarchy of gene expression states is multi-dimensional, 2D representations necessarily distort their true organization. Therefore, while 2D representations of the SPRING plots were used for visualization throughout the study, all quantitative analyses were carried out on the underlying cell nearest-neighbor graph, which accurately captures the high-dimensional nature of the data.

With cells colored according to the expression of lineage-specific marker genes (Fig. 1, Panel c) (ref to source/list of the marker genes), the broad structure of the SRING plot becomes apparent. The cells organize into an undifferentiated core, from which seven distinct branches emerge corresponding to differentiating progenitors of the granulocytic, monocytic, dendritic, lymphoid, megakaryocytic, basophilic, and erythroid lineages. As expected, in fetal (FL) and Epo-stimulated hematopoiesis (eBM), the erythroid branch was significantly expanded compared with basal hematopoiesis (bBM). All the data reported here is made available through an interactive web-based tool, which allows users to explore gene expression on these graphs.

Example 2: Defining the erythroid differentiation trajectory by population balance analysis (PBA)

Each of the three experiments captures cell transcriptomes representing a continuum of all stages of differentiation, from MPPs to lineage-restricted progenitors. To provide a formal basis for ordering cells along their differentiation trajectories, Population Balance Analysis (PBA) was used. PBA is a novel computational approach. PBA orders cells along their differentiation progression by assigning each cell a predicted probability of committing to each of the seven terminal fates. Although they constitute formal predictions of cell fate, the predicted fate probabilities can also be intuitively understood to quantify the distance of any cell from each of the branch points leading to the seven terminal fates. When applied to the bBM data, the PBA predictions are consistent with previous functional assays on fractionated HPC sub-populations. Although PBA can be used to predict regulators of all seven lineage in this data set, here the focus is predominantly on erythropoiesis.

To isolate the erythroid trajectory for detailed study, an MPP-to-erythroid axis in each of the three experiments was defined by ordering cells based on their graph distance from the MPPs, and keeping only cells for which the probability of erythroid fate increases

with distance (Fig. 1, Panel d). These cells were used to create a smoothed time series for every gene along early erythroid differentiation, in a manner akin to previously known pseudotemporal ordering algorithms. To establish confidence in the ordering of cells along the erythroid trajectory, the expression of known erythroid regulators (Fig. 1, Panel e) was examined. It was found that the unbiased ordering of scRNA-Seq data spontaneously recapitulates all of the events examined: transcriptional regulators that function in hematopoietic stem cells/MPP, like GATA2, Tal1, Ldb1, and PU.1, are found expressed at the MPP starting point; GATA1 is then induced and increases rapidly , concurrent with suppression of PU.1, a reciprocal event in the specification of the megakaryocytic and erythroid lineages. The Epo receptor, EpoR , is rapidly induced early in the progression and maintains a high plateau, rising to a further peak with its target, the transferrin receptor (Tfrc), just prior to ETD (represented at the very end of the plot in Fig. 1, Panel e as a Kit-low phase). Additional established events in early erythropoiesis were also observed, including an early peak in GATA2 that is rapidly suppressed with increasing GATA1, reflecting the GATA switch, while other key erythropoietic transcription factors (TFs), Klf1, Tal1, FOG1 (Zfpm1) and Ldb1, are induced and/or continue to be expressed at high levels throughout the progression. CD34 and Kit, markers of MPP and early progenitors , are expressed initially, and are repressed with their distinct dynamics. Taken together, the temporal ordering of the single cell transcriptomes accurately reflects known events of early erythropoiesis.

Example 3: Gene expression dynamic patterns reveal a prominent amplification module in early erythropoiesis

Having inferred a putative transcription ordering of early erythropoiesis, over 4,000 genes that vary significantly along the MPP-to-erythroid axis were identified. Focusing initially on the bBM data, the gene expression profiles revealed large groups of genes showing similar, stable expression, punctuated by rapid concurrent changes in gene expression (Fig. 2, Panel a). This suggested that early erythropoiesis might naturally partition into distinct stages.

To define stages of erythropoiesis, regions with low frequency of gene inflexion (on/off) on the MPP-to-erythroid axis (Fig 2, Panel b) and fate branch points along the differentiation trajectory, (Fig. 1, Panels c and d) were examined. Fate branch points were formally found as regions where PBA-predicted fate probabilities increase for non-erythroid

fates (Fig 2, Panel c). From these metrics, the erythroid trajectory was partitioned into five stages (Fig. 2, Panels a-e), starting with multipotent MPPs and ending with ETD. The existence of three intermediate stages represents the first key prediction of the analysis, for which experimental support is provided in the following sections.

The three intermediate stages were termed: (1) erythroid-basophil-megakaryocytic progenitors (EBMP), (2) early erythroid progenitors (EEP), and (3) committed erythroid progenitors (CEP). EBMPs include cells passing branch points to megakaryocytic and basophil lineages, and were thus predicted to be uncommitted. The EEP stage is a narrow bottleneck just past the final non-erythroid fate branch point, in which gene expression changes rapidly (Fig. 2, Panels b, c, and e). The CEP contains the majority of pre-ETD erythroid progenitors, and it terminates with a sharp transcriptional change leading to ETD with the onset of Tfrc expression (Fig. 2, Panels a-c). Representative erythroid genes that mark each of the specific stages are shown in Fig. 2d.

To assess biological function and transcription factor target enrichment in the EBMP, EEP and CEP stages, the gene expression profiles were temporally clustered. A gene set enrichment analysis (GSEA) of the dynamic gene clusters was carried out (Fig. 2f-h and Extended Data Fig. 2). Strikingly, the most dominant dynamic clusters were of genes specifically upregulated at the CEP stage (represented by Car1, Nfia in Fig. 2, Panel f). Consistent with the population expansion at this stage, these clusters are enriched for cell cycle and growth-related genes, including mTOR signaling, nucleotide metabolism, RNA processing, oxidative phosphorylation, DNA replication, and mitochondrial components. These annotation results suggested that CEPs may act as an early "amplification" module for significant population expansion. Consistent with this hypothesis, CEPs are the most abundant cells in early erythropoiesis.

Genes expressed only in the MPP stage were enriched for non-erythroid lineage terms, while ETD-specific genes were enriched for erythroid pathway components such as heme metabolism, and for targets of the canonical erythroid TFs Gata1, Tal1, and Klf1, although the TFs themselves showed initial upregulation much earlier. This analysis predicts several new epigenetic and transcriptional regulators of erythroid progression: KDM5a/b, ZFX, XRN2, FOXM1, CREM, AFX (FOXO4) (Fig. 2, Panel g). Curiously, although GATA1 itself is expressed essentially throughout the erythroid trajectory (a prediction of the transcriptional ordering that is experimentally validated below), its targets do not rise

strongly prior to the ETD. However, some weak GATA1 activity may be present even very early on: within the MPP compartment the genes correlating most strongly with PBA-predicted erythroid fate were enriched as targets of Tal1, GATA1, GATA2, EVI1 (Mecom) and Myb, as well as targets of regulators with fewer known direct links to erythropoiesis, Mtf2, Rela, Ep300 and Hif1a.

Example 4: Validation of the SPRING plot and PBA predictions

To test the above predictions, a PU.1-GFP reporter mouse and a cell-surface marker expressed by progenitors with an erythroid differentiation bias, CD55 were examined. The SPRING plot shows that CD55+ cells and PU.1-expressing cells occupy extensive non-overlapping regions that emanate in opposite directions from the MPP. No overlap was found between PU.1-GFP expressing cells and cells positive for CD55+ by flow cytometry, thus confirming the gross morphology of the SPRING plot, and suggesting that the early erythroid trajectory lies within the CD55+ fraction of Kit+ cells (Fig. 3, Panel a).

For detailed analysis, a flow cytometric sorting strategy was developed and guided by the SPRING plot expression patterns of alpha integrins . Cell surface markers CD150 and CD105, used previously to identify CFUe12, were combined with Itga2b, Itga6 and the transferrin receptor (Tfrc, CD71) to subdivide Kit+CD55+ bone-marrow into five subpopulations, P1 to P5 (Fig. 3, Panel b). Using qRT-PCR for genes that mark specific locations within the SPRING plot, each of P1 to P5 maps onto a distinct region of the plot, with P1, P2 and P5 forming the principal regions of the erythroid trajectory, and P3 and P4 marking the basophilic and megakaryocytic branches, respectively (Fig. 3, Panel c). Other cell fates were within the CD55- zone of the plot (Fig. 3, Panel c).

Next, this strategy was used to examine the differentiation potential of each of the sorted populations, and by extension, the fate potential of their corresponding regions on the SRPING plot. Colony formation assays (Fig. 3, Panel d) and liquid culture (Fig. 3, Panel e) in the presence of a cocktail of cytokines that support differentiation of myeloid and erythroid lineages were used. P1 and P2, which correspond to the CEP and EEP regions, respectively, both gave rise to erythroid colonies, confirming their location on the erythroid trajectory. No other subset gave rise to erythroid colonies, suggesting that P1 and P2 contain all unipotential erythroid progenitors in the bone-marrow. Of interest, P1 and P2 differ in their colony morphology: P1 colonies were unifocal, maturing on day 3 (CFUe), but also included larger unifocal colonies that matured later, up to day 7 (Fig. 3d). By contrast, P2

colonies were multifocal, maturing on day 4 (late BFUe), or larger multifocal colonies maturing later (early BFUe). Thus, the molecular stage of progenitors, either EEP or CEP, determines their ability to form either multifocal or unifocal colonies, and is somewhat less rigidly related to the time it takes for the colony to complete erythroid maturation.

The P5 subset gave rise to granulocytic/monocytic (GM), megakaryocytic and mixed myeloid colonies, in agreement with its location in the EBMP and MPP portions of the SPRING plot. P4 was highly enriched for megakaryocytic colonies . Liquid culture of the sorted populations (Fig. 3, Panel e) largely recapitulated the results of colony formation assays. In addition, it showed that the progeny of P3 cells are positive for FceRI, suggesting a basophilic fate, in agreement the mapping of P3 to the basophilic branch of the SPRING plot. P2 cells cultured for 7 days contained CD41+ and FceRI+ progeny, in agreement with the least mature cells within this subset originating from the early bottleneck region, where cells are predicted to have substantial residual probabilities for the basophilic and megakaryocytic fates. CD55- cells, as expected from their location in the GM region of the SPRING plot, gave rise principally to GM colonies and to cells with GM markers in liquid cultures.

Expression of canonical TFs in each of the sorted populations was consistent with prediction (Fig 1, Panel e). Taken together, these analyses validate the SPRING plot structure, the extracted erythroid trajectory, and PBA predictions

Example 5: Novel regulators of early erythroid progenitors

Next, EEP and CEP genes that encode cell-surface receptors with known ligands were screened for which could be tested for a potential regulatory effect. Three such receptors were identified: ryk, mst1r and IL-17ra. Ryk belongs to the receptor tyrosine kinase (RTK) family but has an inactive kinase domain and functions in non-canonical Wnt signal transduction. Mst1r (macrophage stimulating 1 receptor/ RON kinase) is an RTK expressed in multiple tissues and tumors. It was found to associate with the EpoR in a CFUe culture system, but could not be activated directly by its ligand, Macrophage Stimulating Protein (MSP/mst1), in that context. IL-17ra is one of several receptors for the IL-17 proinflammatory cytokines, which have been reported to be broadly inhibitory on bone-marrow progenitors. However, the expression of an IL-17 receptor by erythroid progenitors had not been documented.

Here, IL-17ra is expressed in the EEP region of the bBM SPRING plot (Fig. 4, Panel a) and its corresponding P2 subset (Fig. 4, Panel b), and to a lesser extent in the CEP/P1 region, in addition to its expression by non-erythroid cells. Both Ryk and mst1r are expressed more selectively, principally within the CEP/P1 region of the erythroid trajectory (Fig. 4, Panels a and b). Wnt5a, MSP and IL-17a were used to stimulate Ryk, mst1r and IL-17ra respectively, and measured their interaction with Epo, an essential erythroid cytokine, using colony formation assays (Fig. 4, Panel c) . Epo concentration in blood ranges from ~5-10 mU/ml in the basal state, to 10,000 mU/ml in extremely severe anemia . The addition of MSP to FL in the presence of mildly elevated Epo concentrations (50 mU/ml, characteristic of mild anemia) was equivalent to a 10-fold increase in Epo concentration, generating over 2 fold the number of CFUe (day 3 unifocal) colonies. However, in the context of high Epo (>100 mU/ml), the addition of MSP was inhibitory. MSP-mediated inhibition was also seen in earlier FL progenitors and in BM progenitors of all stages. Unlike MSP, Wnt5a was a consistent and potent inhibitor of all erythroid colony formation in both FL and BM. Finally, IL-17a had a striking potentiation of BM CFUe colony formation throughout the Epo dose-response curve, giving rise to 4-fold more colonies at lower Epo (50mU /ml), and increasing the maximal colony formation number at high Epo by 50%. It had a milder stimulatory effect in earlier progenitors that did not persist at the higher Epo doses, and no effect in FL progenitors (Fig. 7). In summary, MSP, Wnt5a and IL-17a may provide powerful modulation of pre-ETD erythroid progenitors, in a manner that is highly dependent on their developmental stage and background state of progenitor stimulation (stress v. basal).

Example 6: Synchronization in S phase at the switch from early erythropoiesis to ETD

GSEA analysis shown in Figure 2 indicates that DNA replication and S phase genes are prominent during the CEP stage (Fig. 2, Panels f and h). Broadly, these genes fall into two dynamic patterns: those whose expression within the CEP stage is stable (Fig. 2, Panel f, Nfia cluster;, and genes whose expression is ramped up with progression through the CEP stage, rising to a sharp peak just prior to the transition into ETD (Fig. 2, Panel f, E2F4 cluster) .The latter dynamic suggests the possibility that its genes are mechanistically relevant to the activation of the ETD program. To examine this further, an unbiased analysis using the Whitfield database (*Mol Biol Cell* **13**, 1977-2000, 2002) of genes with cell cycle-phase –specific expression was pursued. The genes of each cell cycle phase were ordered by the timing of their peak expression along the MPP to Ery progression (Fig 5, Panel a).This

shows a notable group of G1/S and S phase genes peaking simultaneously, just prior to the ETD transition (marked by a dashed yellow line); a large group of G2 and G2/M genes also peaked simultaneously, just following the ETD transition. A line plot of the mean expression of all genes from each cell cycle phase (Fig. 5, Panel b) confirms a peak of G1/S and S phase genes simultaneous with that of the sharp expression peak of the TF Klf1, and just preceding the rapid induction of Tfrc (CD71), a marker of ETD activation. The G1/S and S phase peaks are followed closely by G2 and G2/M peaks. Since the cells are ordered according to their total gene expression state, likely dominated by their differentiation stage, the orderly sequential pattern of G1/S, S, G2 and G2/M peaks suggests that transition into the ETD is synchronized with the cell cycle phase, specifically, with G1/S or S phase.

In addition, examination of genes associated with each cell cycle phase shows that different subsets of regulators appear to dominate different segments of the erythroid trajectory (Fig. 5, Panel a). For example, in the case of cyclin D, a G1 cyclin that promotes S phase entry, the ccnd2 isoform dominates the EEP and CEP stages, to be replaced with the ccnd3 isoform at the ETD transition (Fig. 7). For the E2F TFs, which function as key regulators of S phase genes, E2F6 is stably expressed through the CEP stage, E2F4 is ramped up with progression along the CEP stage, E2F1/7/8 peak with the ETD transition, and E2F2 is induced during the ETD. These patterns raise the possibility that, at each stage of differentiation, a distinct set of regulators drives a differentiation-stage-specific specialized cell cycle program.

In the FL, activation of the ETD coincides with, and is dependent on, a specialized S phase, in which DNA replication rate is faster. The present observations (Fig. 5, Panels a and b) predict a similar interaction between S phase and activation of the ETD in the adult BM. To test this possibility, the cell cycle status of CEP/P1 and EEP/P2 cells were first examined in vivo by pulsing mice with the nucleotide analog bromodeoxyuridine (BrdU) for 30 minutes prior to BM harvesting (Fig. 5, Panel c). BrdU incorporates into DNA of cells that are in S phase during the time of the pulse. The fraction of cells in S phase (BrdU+) increases dramatically in CEP/P1 cells as they begin to upregulate CD71 (Tfrc), in close agreement to the line plots of Tfrc and S phase genes in Fig. 5, Panel b. Of interest, the rate of BrdU incorporation into CEP/P1 cells in S phase was substantially higher following upregulation of Tfrc (CD71hi) (Fig. 5, Panel c, mean S phase BrdU), suggesting that, as in the FL, the S phase that is synchronized with ETD activation is specialized by being faster.

To test whether the synchronization of ETD activation with S phase reflects mechanistic dependence, BM cells were isolated prior to the onset of ETD (Kit+CD55+Lin-CD71lo, Fig. 5, Panels f-h). The cells were cultured in the presence of Epo, which allowed them to activate the ETD, reflected first by the upregulation of CD71, followed by the upregulation of the erythroid cell surface marker Ter119, and induction of the beta-globin gene (Hbb-b1). The addition of the DNA polymerase inhibitor aphidicolin (Aphi) for the first 10 hours of culture, arrested S phase and paused upregulation of CD71, both of which resumed once Aphi was removed. The ensuing ETD activation proceeded with a 10-hour delay in the induction of CD71 and Hbb-b1 (Fig. 5, Panels g and h). These findings show that activation of the ETD is synchronized with, and requires, a specialized, faster S phase.

Example 7: Fetal and stress erythropoiesis: key genetic pathways

The absolute number of Kit+ cells does not change following Epo stimulation. Therefore, expansion of the amplification module is at the expense of non-erythroid outcomes. This raises the possibility that Epo stimulation, either directly or indirectly, alters the MPP differentiation bias, or somehow inhibits non-erythroid outcomes.

Example 8: Growth factor regulators of early erythropoiesis

As described in Example 5, EEP and CEP expressed genes that encode cell-surface receptors with known ligands were screened and then tested for potential regulatory effects. Three such receptors: *Ryk*, *Mst1r* and *Il17ra* were identified.

In bone marrow (BM), *Il17ra* was expressed by EEPs/P2 and to a lesser extent by CEPs/P1, in addition to non-erythroid cells. Both *Ryk* and *Mst1r* are expressed more selectively, though not exclusively, by EEP and CEP cells (Fig. 15, Panel a, and Fig. 16, Panels a and b). Ryk, Mst1r and IL-17Ra were stimulated with their respective ligands, Wnt5a, MSP and IL-17a, using Epo-dependent erythroid colony formation as readout (Fig. 15, Panels b and c, Fig. 7). Epo concentration in blood varies from ~10 mU/ml in health, to 10,000 mU/ml in severe anemia. It was found that addition of MSP to FL in the presence of mildly elevated Epo (50 mU/ml) was equivalent to a 10-fold increase in Epo concentration, generating over 2- fold the number of CFU-e colonies. However, MSP was inhibitory in other contexts, and Wnt5a was a consistent and potent inhibitor of all erythroid colony formation in both FL and BM (Fig. 15, Panel b, Fig. 7). By contrast, IL-17a mediated a potentiation of adult BM CFU-e colony formation throughout the Epo dose-response curve, giving rise to 4-fold more colonies at lower Epo (50mU /ml), and to a ~50% increase in

colony formation when added to maximal levels of Epo (Fig 15, Panels b and c). It had a milder stimulatory effect in BFU-e progenitors, and no effect in FL (Fig. 16).

The stimulatory effect of IL-17a on CFU-e formation was mediated through expression of IL-17Ra, as shown by its absence in BM of IL-17Ra-deleted mice (Fig 15, Panel d), and was also evident in human BM (Fig 15, Panel e). Further, IL-17a stimulation was saturable, with a low EC_{50} (2.0 ± 1.2 ng/ml (60 pM) in mouse, 2.7 ±1.6 ng/ml (81 pM) in human BM), consistent with it being the direct consequence of high affinity-binding of IL-17a to IL-17Ra. Stimulation with IL-17a resulted in rapid phosphorylation of the intracellular signaling mediators Stat3 and Stat5 in CEP and EEP (Fig. 15, Panel f). Further, freshly sorted BM CEP/P1 and EEP/P2 expressed IL-17Ra by western blotting (Fig. 15, Panel g). Taken together, these findings suggest previously unknown complex regulatory modulation of EEP and particularly of CEP through the expression of a number of growth factor receptors new to erythropoiesis.

Methods:

Growth factor perturbations of erythroid colony formation: CFU-e and BFU-e colony formation assays in MethoCult (M3234 STEMCELL Technologies) were carried out on either freshly isolated bone marrow or on embryonic day 13.5 fetal liver cells from Balb/cJ mice. The following growth factors were tested: MSP/MST1 (R&D systems; CAT #6244-MS-025), Recombinant Human/Mouse Wnt-5a (R&D systems; CAT #645-WN-010) and Recombinant Murine IL17 (IL-17A) (PeproTech; CAT #210-17). In each experiment, a range of Epo concentrations was tested, with or without added additional growth factors (MSP, Wnt5a or IL-17A) as indicated in Fig. 5 and in Extended Data Fig. 12. In addition to Epo, IL-3 (10ng/mL) and SCF (50ng/ mL) were added to the MethoCult in the case of BFU-e assays. Each condition was tested in quadruplicates, in at least 2 separate experiments. Colonies were scored on day 3 (for CFU-e), day 4 (for late BFU-e) and on day 7 (for early BFU-e) following staining with diaminobenzidine, to highlight hemoglobin expression.

IL17RA -deleted mice: To generate the IL-17RA -deleted line, IL-17RA flox/+ mice9 were bred with CMV-Cre mice (#003465, JAX lab). The generation of il17ra del allele in the F1 generation of il17raflox/+ × CMV-Cre mating pairs were screened by PCR of tail DNA. To remove the CMV-Cre allele present in the F1 generation, IL-17RA del/+; CMV-Cre+/– mice were outcrossed with B6 mice.

Colony-formation assays with human bone-marrow: Human bone-marrow mononuclear cells (MNCs) (85,000 cells, STEMCELL Technologies 70001.1) were mixed with 1 ml MethoCult (STEMCELL Technologies H4230) supplemented with either EPO (0.05U/ ml), and in the presence or absence of IL-17a (R&D systems 7955-IL-025). CFUe colonies were scored from triplicate plates on day 7.

Flow cytometric sorting for P1 to P5 subsets: Bone marrow cells from adult BALB/cJ male or female mice, ages 8-12 weeks, were lineage-depleted using the Mouse Streptavidin RapidSpheres Isolation Kit (STEMCELL Technologies [Cat# 19860A]), with the following biotinylated antibodies:

anti-CD11b (Clone M1/70 [#557395], BD Biosciences)
anti-Ly-6G and Ly-6C (Clone RB6-8C5 [#553125], BD Biosciences)
anti-CD4 (Clone RM4-5 [#553045], BD Biosciences)
anti-CD8a (Ly-2) (Clone 53-6.7 [#553029], BD Bioscience)
anti-CD19 (Clone 1D3 [#553784], BD Biosciences)
anti-TER119 (Clone TER119 [#553672], BD Biosciences).

Lineage-depleted cells were then labeled with the following antibodies in the presence of 1% rat serum:

streptavidin Alexa Fluor 488 (Molecular Probes), to mark lineage-positive cells
CD117- APC Cy7 (Clone 2B8 [#105826], Biolegend)
TER119-BUV395 (Clone TER-119 [#563827], BD Biosciences)
CD71- PE Cy7 (Clone RI7217 [#113812], Biolegend)
CD55-AF647 (Clone RIKO-3 [#131806], Biolegend)
CD105-PE (Clone MJ7/18 [#120408], Biolegend)
CD150-BV650 (Clone TC15-12F12.2 [#115931], Biolgened)
CD41-BV605 (Clone MWReg30 [#133921], Biolegend)
CD49f (=itga6) – BV421 (Clone GoH3 [#313624], Biolegend)

Following washes, cells were re-suspended in DAPI-containing buffer and sorting was performed on BD FACSAria II with a 100 µ nozzle. Sorted populations were defined as in Fig. 14.

qRT-PCR on sorted populations: RNA was prepared from sorted cell subsets using the RNeasy Micro Kit (QIAGEN; CAT# 74004) or TRIzol reagent (Ambion; CAT# 15596026), and measured with RiboGreen RNA reagent kit (Thermo Scientific) on the 3300

NanoDrop Fluorospectrometer. cDNA was synthesized using the same amount of input RNA for all samples in a parallel reaction, using the Super Script III first-strand synthesis system for RT-PCR (Invitrogen) with random hexamer primers. The ABI 7300 sequence detection system, TaqMan reagents and TagMan MGB probes (Applied Biosystems, San Diego, CA) were used following the manufacturer's instructions. qPCR was carried on 4 serial dilutions of each cDNA sample, and the linear part of the template dilution/signal response curve was used to calculate relative mRNA concentrations following normalization to β-actin, using the ΔCt method. The following TagMan MGB probes were used: Mst1r (Mm00436382_m1), Ryk (Mm01238551_m1), IL17ra (Mm00434214_m1), Mt2 (Mm00809556_s1)

Western Blot Analysis: Bone marrow cells were sorted as above, except that the P1 population was further subdivided into CD71med and CD71hi subsets. For negative controls, 3T3-L1 cells were used. For positive controls, 3T3-L1 cells were transduced with the MICD4-GATA1 retrovirus. Cell pellets were snap-frozen in liquid nitrogen following the sort. Cell lysates were quantified by the BCA Protein Assay Kit (Pierce) and separated by SDS-PAGE gel electrophoresis. PVDF membranes were probed with antibodies against GATA1 (N6, sc-265, Santa Cruz), β-actin (ab8227, abcam), MCM5 (Bethyl Laboratories, Inc., A300-195A-M), MCM6 (Bethyl Laboratories, Inc., A300-194A), MCM2 (Bethyl Laboratories, Inc., A300-191A), PCNA (PC10) (Santa Cruz, sc-56), IL-17RA/IL-17R (R&D Systems, AF448). Western blot membranes were quantified using the BIORAD Imaging system and Image Lab software.

Intracellular signaling by Stat3 and Stat5: Freshly harvested bone-marrow cells were enriched for Lin-Ter119- cells using magnetic beads. The enriched cells were incubated a cytokine-free, low serum medium (IMDM with 2% FCS) for 3 hours. EPO (0.5U/ml) only, IL-17a (20ng/ml) only, or EPO (0.5U/ml) and IL-17a (20ng/ml) were then added to the medium for either 30 or 60 minutes. Cells were harvested, washed with PhosphoWash Buffer 11, stained with LIVE/DEAD kit (Invitrogen), fixed and permeabilized with Cytofix/Cytoperm Buffer (BD 554722) supplemented with 1mM Sodium Orthovanadate (Sigma 450243-10G), 1mM β-glycerophosphate (Sigma G9422-10G) and 1ug/ml Microcystin (EMD Millipore 475815-500UG), and Perm/Wash Buffer I (BD 557885), and frozen in freezing medium (90% FCS, 10% DMSO, 1mM Sodium Orthovanadate, 1mM β-glycerophosphate and 1ug/ml Microcystin). When thawed, cells were re-fixed and permeabilized, incubated with 5% milk and 200ug/ml Rabbit IgG (modified from Porpiglia

et al., PLoS Biol. 2012), and stained with p-Stat3-AF488 (B-7) (Santa Cruz sc-8059 AF488), p-Stat5-AF647 (pY694) (BD Bioscience 612599), CD71-PE/Cy7 (Biolegend 113812), CD55-PE (Biolegend 131804), CD105-Pacific Blue (Biolegend 120412), CD150-BV650 (Biolegend 115931), CD49f (Itga6)-PE/Dazzle 594 (Biolegend 313626), CD41-BV605 (Biolegend 133921), CD117 (Kit)-APC/H7 (BD Bioscence 560185), Strepavidin-AF700 (Invitrogen S21383) and DAPI. Analysis was on an LSRII FACS analyzer.

Incorporation by Reference

All publications, patents, and patent applications mentioned herein are hereby incorporated by reference in their entirety as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A method of stimulating erythropoiesis in a subject with anemia comprising administering to the subject an agent that binds to MST1r.

2. The method of claim 1, wherein the agent comprises a MSP ligand protein or a fragment thereof.

3. The method of claim 1, wherein the agent comprises an antibody that binds to MST1r or an antigen binding fragment thereof.

4. The method of any one of claims 1 to 3, wherein the subject is an infant.

5. The method of any one of claims 1 to 4, wherein the subject has congenital anemia.

6. The method of any one of claims 1 to 4, wherein the subject has Diamond Blackfan Anemia.

7. The method of any one of claims 1 to 4, wherein the subject has a cancer-associated anemia.

8. The method of claim 7, wherein the cancer is breast, prostate, lung, skin, colorectal, cervical, pancreatic, ovarian, lymphoma, leukemia, liver, testicular, and brain cancer .

9. The method of any one of claims 1 to 8, further comprising administering to the subject erythropoietin or an erythropoietin derivative.

10. The method of claim 9, wherein the erythropoietin or erythropoietin derivative is asialoerythropoietin, N-deglycosylated erythropoietin, O-deglycosylated erythropoietin, erythropoietin with reduced carbohydrate content, erythropoietin with altered glycosylation patterns, erythropoietin with carbohydrates oxidized then reduced, arylglyoxal-modified erythropoietin, alkylglyoxal-modified erythropoietin, 2,3-butanedione-modified erythropoietin, cyclohexanedione-modified erythropoietin, biotinylated erythropoietin, N-alkylated-lysyl-erythropoietin, glucitolyl lysine erythropoietin, alpha-deoxy-alpha-fructosyllysine-erythropoietin, carbamylated erythropoietin, acetylated erythropoietin, succinylated erythropoietin, alpha-carboxyalkyl erythropoietin, nitrated erythropoietin, iodinated erythropoietin.

11. A method of stimulating erythropoiesis in a subject with anemia comprising administering to the subject an agent that binds to IL-17ra.

12. The method of claim 11, wherein the agent comprises a protein selected from an IL-17a protein or a fragment thereof, an IL-17f protein or fragment thereof, and a heterodimeric IL-17a/f protein or fragment thereof.

13. The method of claim 11, wherein the agent comprises an antibody that binds to IL-17ra or an antigen binding fragment thereof.

14. The method of any one of claims 11 to 13, wherein the subject is an infant.

15. The method of any one of claims 11 to 14, wherein the subject has congenital anemia.

16. The method of any one of claims 11 to 14, wherein the subject has Diamond Blackfan Anemia.

17. The method of any one of claims 11 to 14, wherein the subject has a cancer-associated anemia.

18. The method of claim 17, wherein the cancer is selected from breast, prostate, lung, skin, colorectal, cervical, pancreatic, ovarian, lymphoma, leukemia, liver, testicular, and brain cancer .

19. The method of any one of claims 11 to 18, further comprising administering to the subject erythropoietin or an erythropoietin derivative.

20. The method of claim 19, wherein the erythropoietin or erythropoietin derivative is asialoerythropoietin, N-deglycosylated erythropoietin, O-deglycosylated erythropoietin, erythropoietin with reduced carbohydrate content, erythropoietin with altered glycosylation patterns, erythropoietin with carbohydrates oxidized then reduced, arylglyoxal-modified erythropoietin, alkylglyoxal-modified erythropoietin, 2,3-butanedione-modified erythropoietin, cyclohexanedione-modified erythropoietin, biotinylated erythropoietin, N-alkylated-lysyl-erythropoietin, glucitolyl lysine erythropoietin, alpha-deoxy-alpha-fructosyllysine-erythropoietin, carbamylated erythropoietin, acetylated erythropoietin, succinylated erythropoietin, alpha-carboxyalkyl erythropoietin, nitrated erythropoietin, iodinated erythropoietin..

21. A method of stimulating erythropoiesis in a subject with anemia comprising administering to the subject an agent that inhibits the binding of WNT5a to RYK.

22. The method of claim 21, wherein the agent comprises a polypeptide fragment of WNT5a.

23. The method of claim 21, wherein the agent comprises a polypeptide fragment of RYK.

24. The method of claim 21, wherein the agent comprises an antibody that specifically binds to WNT5a or an antigen binding fragment thereof.

25. The method of claim 21, wherein the agent comprises an antibody that specifically binds to RYK or an antigen binding fragment thereof.

26. The method of any one of claims 21 to 25, wherein the subject is an infant.

27. The method of any one of claims 21 to 26, wherein the subject has congenital anemia.

28. The method of any one of claims 21 to 26, wherein the subject has Diamond Blackfan Anemia.

29. The method of any one of claims 21 to 26, wherein the subject has a cancer-associated anemia.

30. The method of claim 29, wherein the cancer is breast, prostate, lung, skin, colorectal, cervical, pancreatic, ovarian, lymphoma, leukemia, liver, testicular, and brain cancer.

31. The method of any one of claims 21 to 30, further comprising administering to the subject erythropoietin or an erythropoietin derivative.

32. The method of claim 31, wherein the erythropoietin or erythropoietin derivative is asialoerythropoietin, N-deglycosylated erythropoietin, O-deglycosylated erythropoietin, erythropoietin with reduced carbohydrate content, erythropoietin with altered glycosylation patterns, erythropoietin with carbohydrates oxidized then reduced, arylglyoxal-modified erythropoietin, alkylglyoxal-modified erythropoietin, 2,3-butanedione-modified erythropoietin, cyclohexanedione-modified erythropoietin, biotinylated erythropoietin, N-alkylated-lysyl-erythropoietin, glucitolyl lysine erythropoietin, alpha-deoxy-alpha-fructosyllysine-erythropoietin, carbamylated erythropoietin, acetylated erythropoietin, succinylated erythropoietin, alpha-carboxyalkyl erythropoietin, nitrated erythropoietin, iodinated erythropoietin..

33. A method of inhibiting erythropoiesis in a subject with erythrocytosis comprising administering to the subject an agent that binds to MST1r.

34. The method of claim 33, wherein the agent comprises a MSP ligand protein or a fragment thereof.

35. The method of claim 33, wherein the agent comprises an antibody that binds to MST1r or an antigen binding fragment thereof.

36. The method of any one of claims 33 to 35, wherein the subject has primary erythrocytosis.

37. The method of any one of claims 33 to 35, wherein the subject has secondary erythrocytosis.

38. The method of any one of claims 33 to 35, wherein the subject has polycythemia vera.

39. The method of claim 38, wherein the subject is further administered a Jak2 inhibitor.

40. The method of claim 39, wherein the Jak2 inhibitor is ruxolitinib, fedratinib, tofacitinib, baricitinib (INCB039110), lestaurtinib (CEP701), pacritinib (SB1518), XL019, AZD1480, gandotinib (LY2784544), BMS911543, fedratinib (SAR302503), decemotinib (V-509), INCB39110, GEN1, GEN2, GLPG0634, NS018, and N-(cyanomethyl)-4-[2-(4-morpholinoanilino)pyrimidin-4-yl]benzamide; or pharmaceutically acceptable salts thereof.

41. A method of inhibiting erythropoiesis in a subject with erythrocytosis comprising administering to the subject an agent that binds to RYK.

42. The method of claim 41, wherein the agent comprises a WNT5a protein or a fragment thereof.

43. The method of claim 41, wherein the agent comprises an antibody that binds to RYK or an antigen binding fragment thereof.

44. The method of any one of claims 41 to 43, wherein the subject has primary erythrocytosis.

45. The method of any one of claims 41 to 43, wherein the subject has secondary erythrocytosis.

46. The method of any one of claims 41 to 43, wherein the subject has polycythemia vera.

47. The method of claim 46, wherein the subject is further administered a Jak2 inhibitor.

48. The method of claim 47, wherein the Jak2 inhibitor is ruxolitinib, fedratinib, tofacitinib, baricitinib (INCB039110), lestaurtinib (CEP701), pacritinib (SB1518), XL019, AZD1480, gandotinib (LY2784544), BMS911543, fedratinib (SAR302503), decemotinib (V-509), INCB39110, GEN1, GEN2, GLPG0634, NS018, and N-(cyanomethyl)-4-[2-(4-morpholinoanilino)pyrimidin-4-yl]benzamide; or pharmaceutically acceptable salts thereof.

49. A method of inhibiting erythropoiesis in a subject with erythrocytosis comprising administering to the subject an agent that inhibits the binding of IL-17a to IL-17ra.

50. The method of claim 49, wherein the agent comprises a polypeptide fragment of IL-17a.

51. The method of claim 49, wherein the agent comprises a polypeptide fragment of IL-17ra.

52. The method of claim 49, wherein the agent comprises an antibody that specifically binds to IL-17a or an antigen binding fragment thereof.

53. The method of claim 49, wherein the agent comprises an antibody that specifically binds to IL-17ra or an antigen binding fragment thereof.

54. The method of any one of claims 49 to 53, wherein the subject has primary erythrocytosis.

55. The method of any one of claims 49 to 53, wherein the subject has secondary erythrocytosis.

56. The method of any one of claims 49 to 53, wherein the subject has polycythemia vera.

57. The method of claim 56, wherein the subject is further administered a Jak2 inhibitor.

58. The method of claim 57, wherein the Jak2 inhibitor is ruxolitinib, fedratinib, tofacitinib, baricitinib (INCB039110), lestaurtinib (CEP701), pacritinib (SB1518), XL019, AZD1480, gandotinib (LY2784544), BMS911543, fedratinib (SAR302503), decemotinib

(V-509), INCB39110, GEN1, GEN2, GLPG0634, NS018, and N-(cyanomethyl)-4-[2-(4-morpholinoanilino)pyrimidin-4-yl]benzamide; or pharmaceutically acceptable salts thereof.

59. A method of determining whether a test agent is an inhibitor of erythropoiesis comprising:

a) forming a test reaction mixture comprising:

an IL-17a protein or an IL-17ra-binding fragment thereof;

an IL-17ra protein or IL-17a-binding fragment thereof; and

a test agent;

b) incubating the test reaction mixture under conditions conducive for the formation of a complex between the IL-17a protein or an IL-17ra-binding fragment thereof and the IL-17ra protein or IL-17a-binding fragment thereof; and

c) determining the amount of the complex in the test reaction mixture;

wherein a test agent that reduces the amount of the complex in the test reaction mixture compared to the amount of the complex in a control reaction mixture is an inhibitor of erythropoiesis.

60. The method of claim 59, wherein the control reaction mixture is substantially identical to the test reaction mixture except that the control reaction mixture does not comprise a test agent.

61. The method of claim 59, wherein the control reaction mixture is substantially identical to the test reaction mixture except that the control reaction mixture comprises a placebo agent instead of a test agent.

61. The method of any one of claims 59 to 61, wherein the test agent is a member of a library of test agents.

62. A method of determining whether a test agent is an inhibitor of erythropoiesis comprising:

a) forming a test reaction mixture comprising:

an MSP ligand protein or an MST1r-binding fragment thereof;

an MST1r protein or MSP ligand-binding fragment thereof; and

a test agent;

b) incubating the test reaction mixture under conditions conducive for the formation of a complex between the MSP ligand protein or an MST1r-binding fragment thereof and the MST1r protein or MSP ligand-binding fragment thereof; and

c) determining the amount of the complex in the test reaction mixture;

wherein a test agent that reduces the amount of the complex in the test reaction mixture compared to the amount of the complex in a control reaction mixture is an inhibitor of erythropoiesis.

63. The method of claim 62, wherein the control reaction mixture is substantially identical to the test reaction mixture except that the control reaction mixture does not comprise a test agent.

64. The method of claim 62, wherein the control reaction mixture is substantially identical to the test reaction mixture except that the control reaction mixture comprises a placebo agent instead of a test agent.

65. The method of any one of claims 62 to 64, wherein the test agent is a member of a library of test agents.

66. A method of determining whether a test agent is an enhancer of erythropoiesis comprising:

a) forming a test reaction mixture comprising:

an WNT5a protein or an RYK-binding fragment thereof;

an RYK-protein or WNT5a ligand-binding fragment thereof; and

a test agent;

b) incubating the test reaction mixture under conditions conducive for the formation of a complex between the WNT5a protein or an RYK-binding fragment thereof and the RYK-protein or WNT5a ligand-binding fragment thereof; and

c) determining the amount of the complex in the test reaction mixture;

wherein a test agent that reduces the amount of the complex in the test reaction mixture compared to the amount of the complex in a control reaction mixture is an enhancer of erythropoiesis.

67. The method of claim 66, wherein the control reaction mixture is substantially identical to the test reaction mixture except that the control reaction mixture does not comprise a test agent.

68. The method of claim 66, wherein the control reaction mixture is substantially identical to the test reaction mixture except that the control reaction mixture comprises a placebo agent instead of a test agent.

69. The method of any one of claims 66 to 68, wherein the test agent is a member of a library of test agents.

70. A method of expanding or propagating erythroid progenitor cells *in vitro* comprising culturing the erythroid progenitor cells with an agent that activates IL-17ra to a cell medium comprising the progenitor cells.

71. The method of claim 70, wherein the agent comprises a protein selected from an IL-17a protein or a fragment thereof, an IL-17f protein or fragment thereof, and a heterodimeric IL-17a/f protein or fragment thereof.

72. A method of expanding or propagating erythroid progenitor cells *in vitro* comprising culturing the erythroid progenitor cells with an agent that inhibits Ryk or Mst1r to a cell medium comprising the progenitor cells.

Figure 1

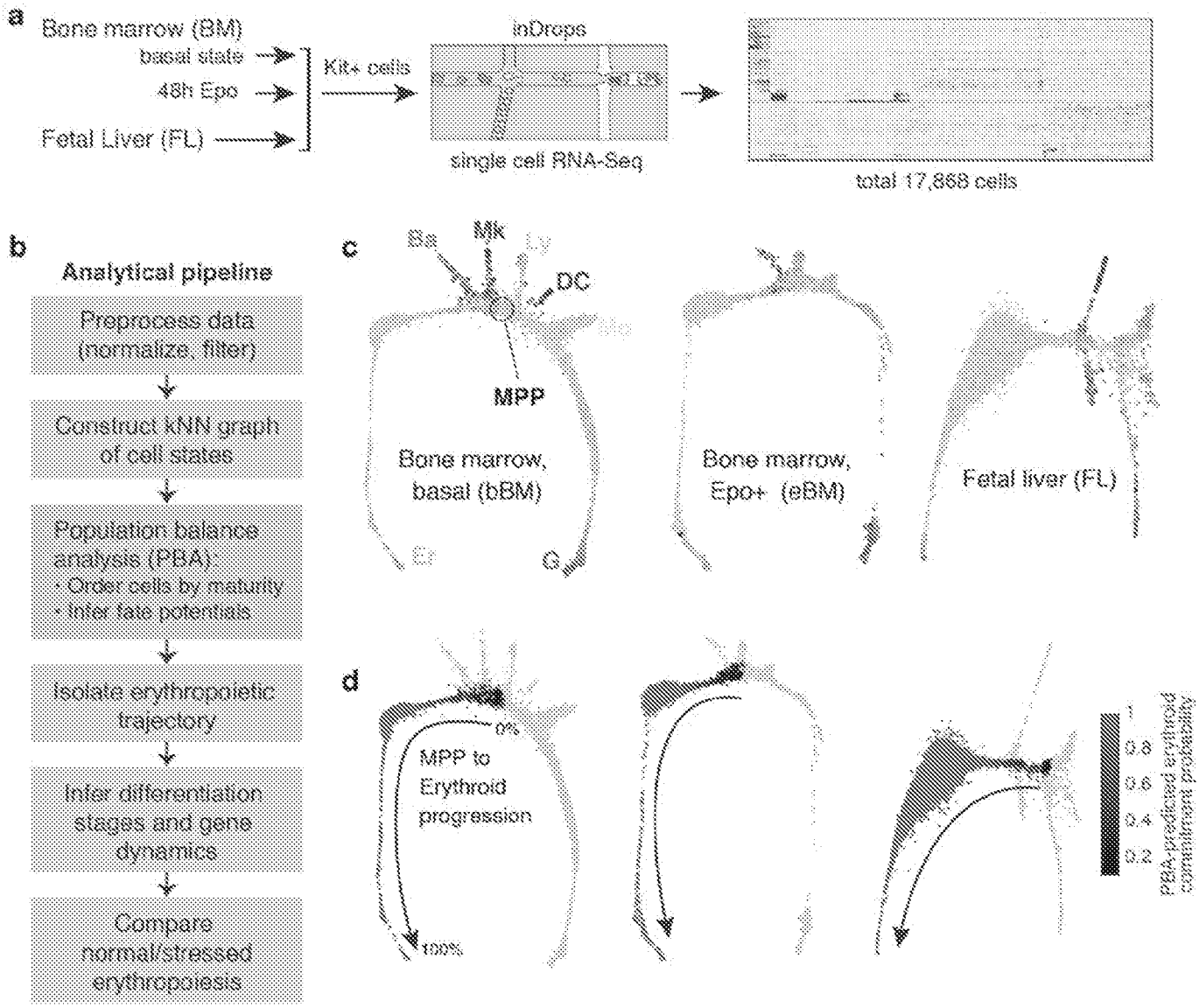
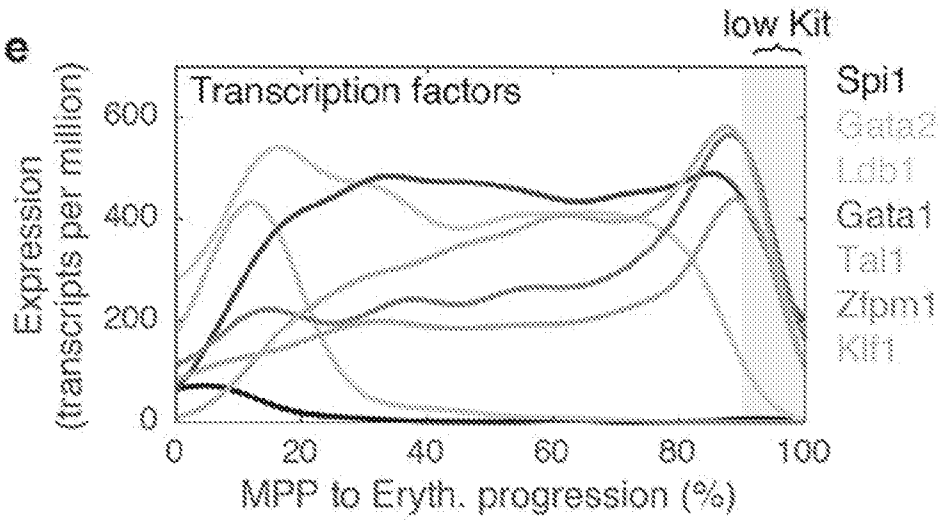

a
Bone marrow (BM)
basal state
48h Epo
Fetal Liver (FL)
Kit+ cells
inDrops
single cell RNA-Seq
total 17,868 cells
b
Analytical pipeline
Preprocess data (normalize, filter)
Construct kNN graph of cell states
Population balance analysis (PBA):
· Order cells by maturity
· Infer fate potentials
Isolate erythropoietic trajectory
Infer differentiation stages and gene dynamics
Compare normal/stressed erythropoiesis
c
Ba
Mk
Ly
DC
Mo
MPP
Bone marrow, basal (bBM)
Er
G
Bone marrow, Epo+ (eBM)
Fetal liver (FL)
d
0%
MPP to Erythroid progression
100%
1
0.8
0.6
0.4
0.2
PBA-predicted erythroid commitment probability

Figure 1(cont.)



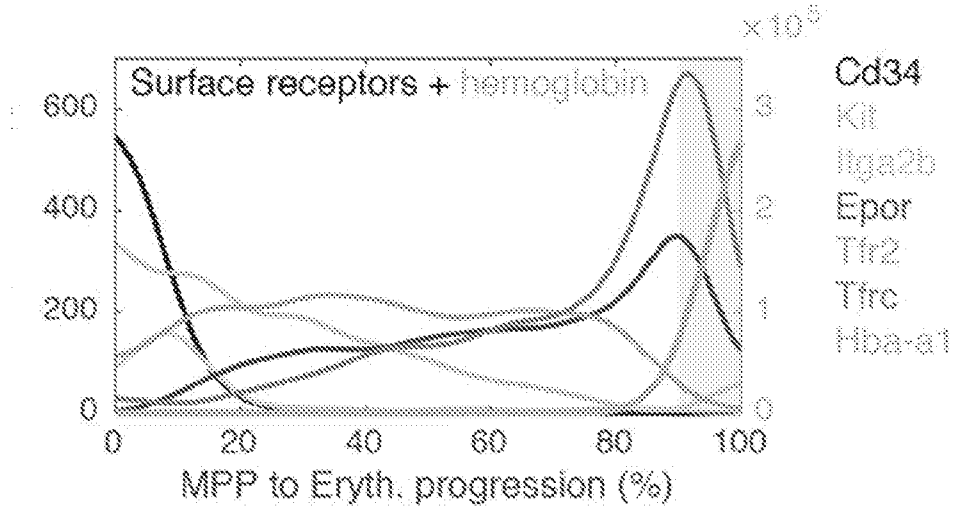


Figure 2

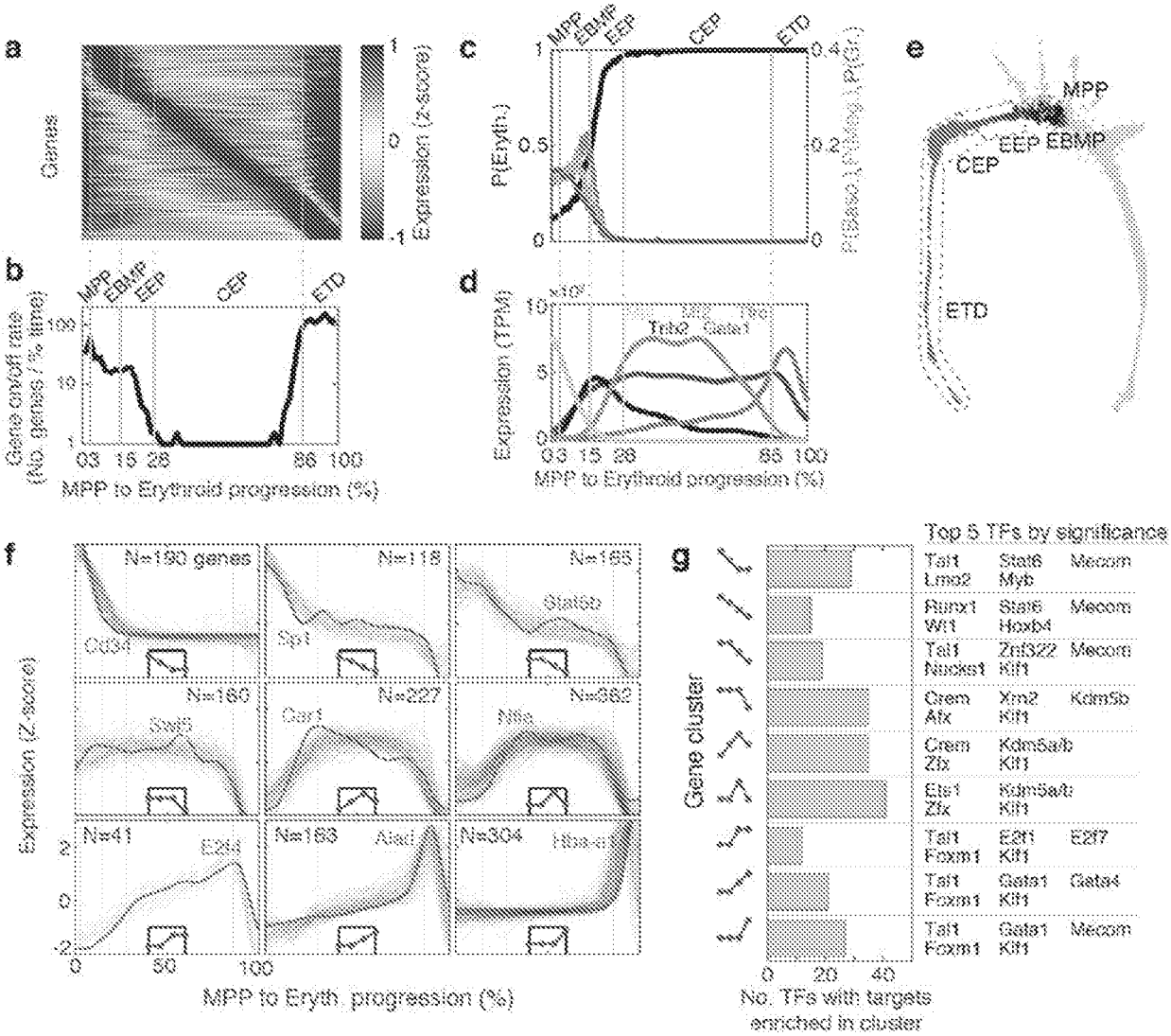

a
Genes
Expression (z-score)
b
MPP
EBMP
EEP
CEP
ETD
Gene on/off rate (No. genes / % time)
MPP to Erythroid progression (%)
c
P(Eryth.)
P(Baso.), P(Meg.), P(Gr.)
d
Expression (TPM)
Trib2
Gata1
MPP to Erythroid progression (%)
e
MPP
EEP
EBMP
CEP
ETD
f
N=190 genes
N=118
N=165
N=160
N=227
N=362
N=41
N=163
N=304
Expression (Z-score)
MPP to Eryth. progression (%)
g
Top 5 TFs by significance
Gene cluster
No. TFs with targets enriched in cluster
Tal1 Lmo2 Stat6 Myb Mecom
Runx1 Wt1 Stat6 Hoxb4 Mecom
Tal1 Nucks1 Znf322 Klf1 Mecom
Crem Afx Xrn2 Klf1 Kdm5b
Crem Zfx Kdm5a/b Klf1
Ets1 Zfx Kdm5a/b Klf1
Tal1 Foxm1 E2f1 Klf1 E2f7
Tal1 Foxm1 Gata1 Klf1 Gata4
Tal1 Foxm1 Gata1 Klf1 Mecom

Figure 2 (cont.)

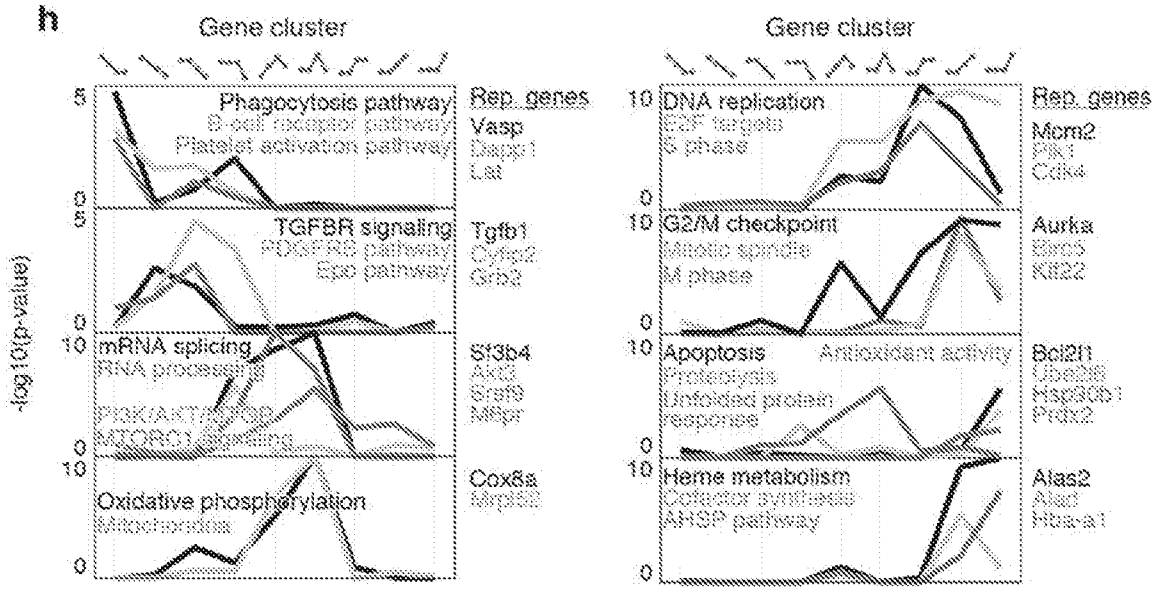


Figure 3

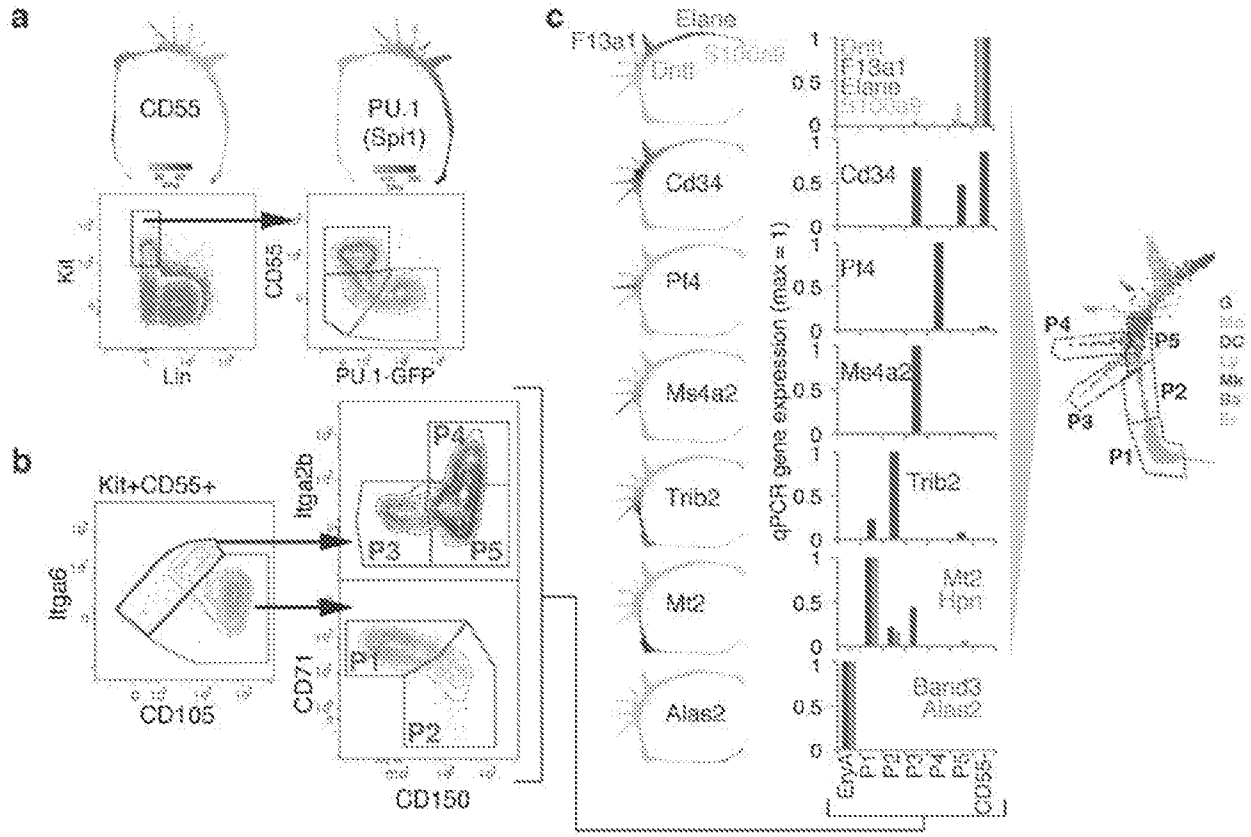
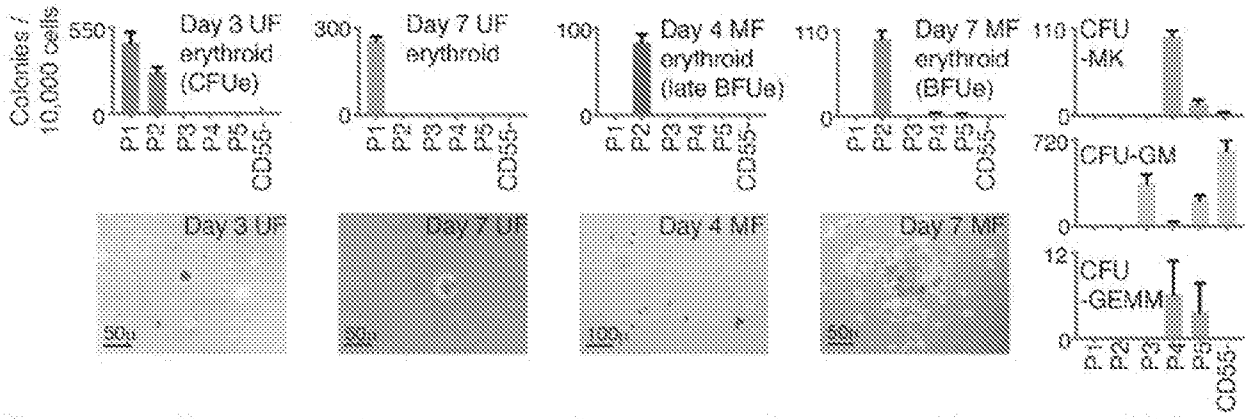

a
CD55
PU.1 (Spi1)
Kit
CD55
Lin
PU.1-GFP
b
Kit+CD55+
Itga2b
Itga6
P4
P3
P5
CD105
CD71
P1
P2
CD150
c
Elane
F13a1
Cd34
Pf4
Ms4a2
Trib2
Mt2
Alas2
qPCR gene expression (max = 1)
Pf4
F13a1
Elane
Cd34
Pf4
Ms4a2
Trib2
Mt2
Hpn
Band3
Alas2
EVA
P1 P2 P3 P4 P5
CD55
P4
P5
P2
P3
P1
CD55

Figure 3 (cont.)

d



e

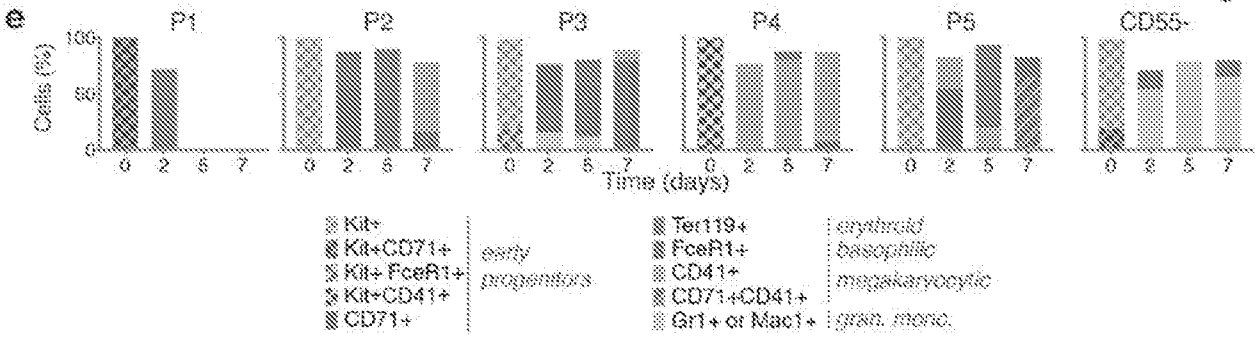


Figure 4

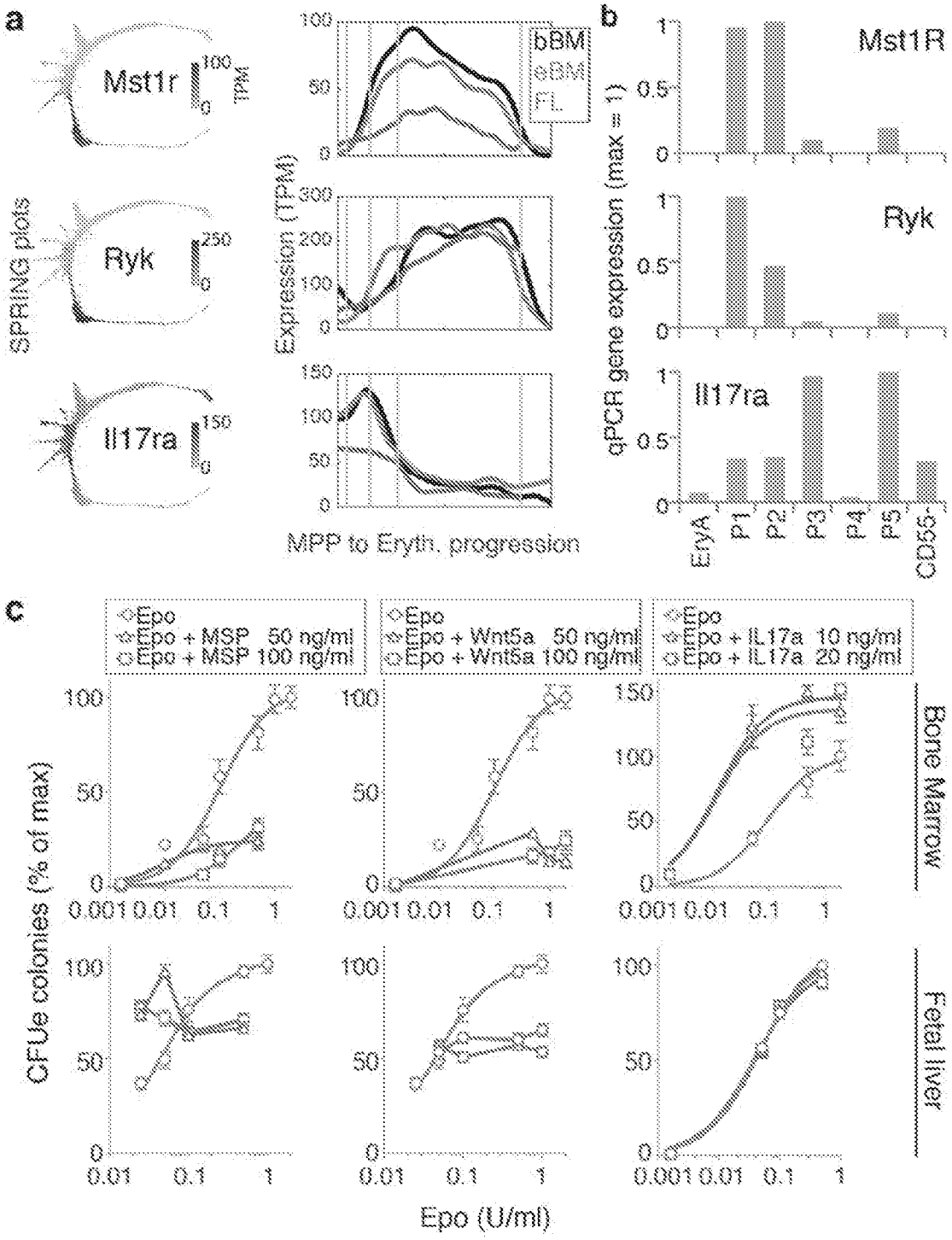

a
Mst1r
Ryk
Il17ra
SPRING plots
TPM
bBM
eBM
FL
Expression (TPM)
MPP to Eryth. progression
b
Mst1R
Ryk
Il17ra
qPCR gene expression (max = 1)
EryA
P1
P2
P3
P4
P5
CD55-
c
Epo
Epo + MSP 50 ng/ml
Epo + MSP 100 ng/ml
Epo + Wnt5a 50 ng/ml
Epo + Wnt5a 100 ng/ml
Epo + IL17a 10 ng/ml
Epo + IL17a 20 ng/ml
CFUe colonies (% of max)
Bone Marrow
Fetal liver
Epo (U/ml)

Figure 5

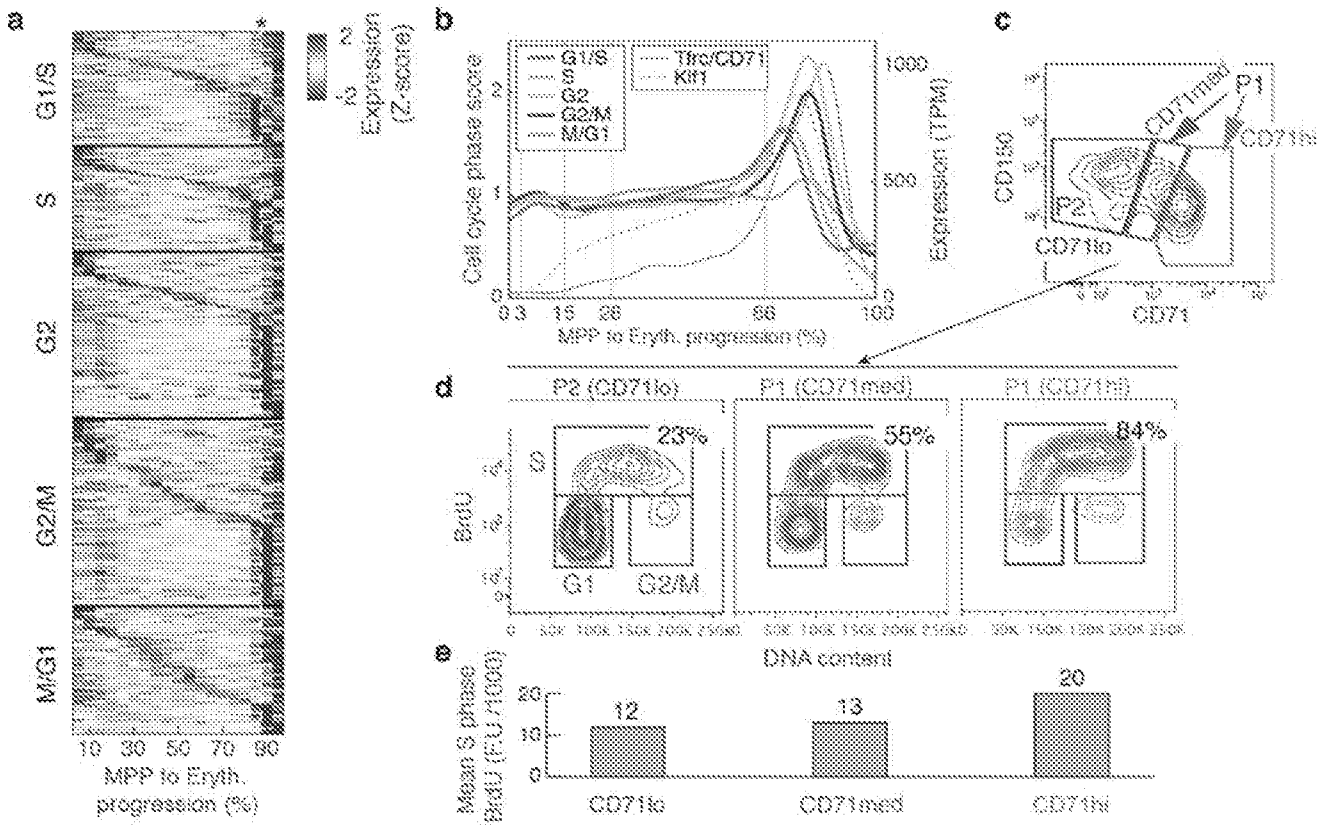

a
G1/S
S
G2
G2/M
M/G1
Expression (z-score)
MPP to Eryth. progression (%)
b
Cell cycle phase score
Expression (TPM)
Tfrc/CD71
Kif1
MPP to Eryth. progression (%)
c
CD150
CD71
P1
P2
CD71med
CD71hi
CD71lo
d
P2 (CD71lo)
P1 (CD71med)
P1 (CD71hi)
23%
55%
84%
BrdU
DNA content
e
Mean S phase BrdU (FU /1000)
12
13
20
CD71lo
CD71med
CD71hi

Figure 5 (cont.)

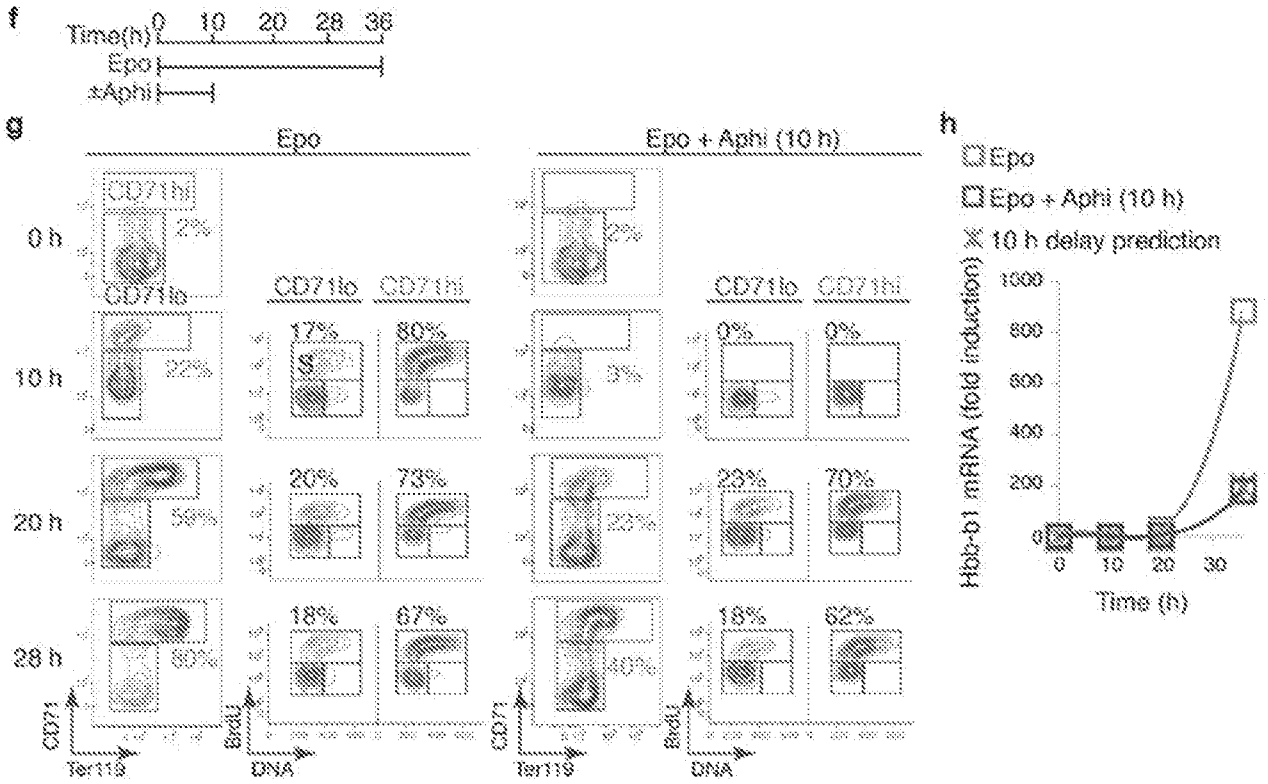


Figure 6

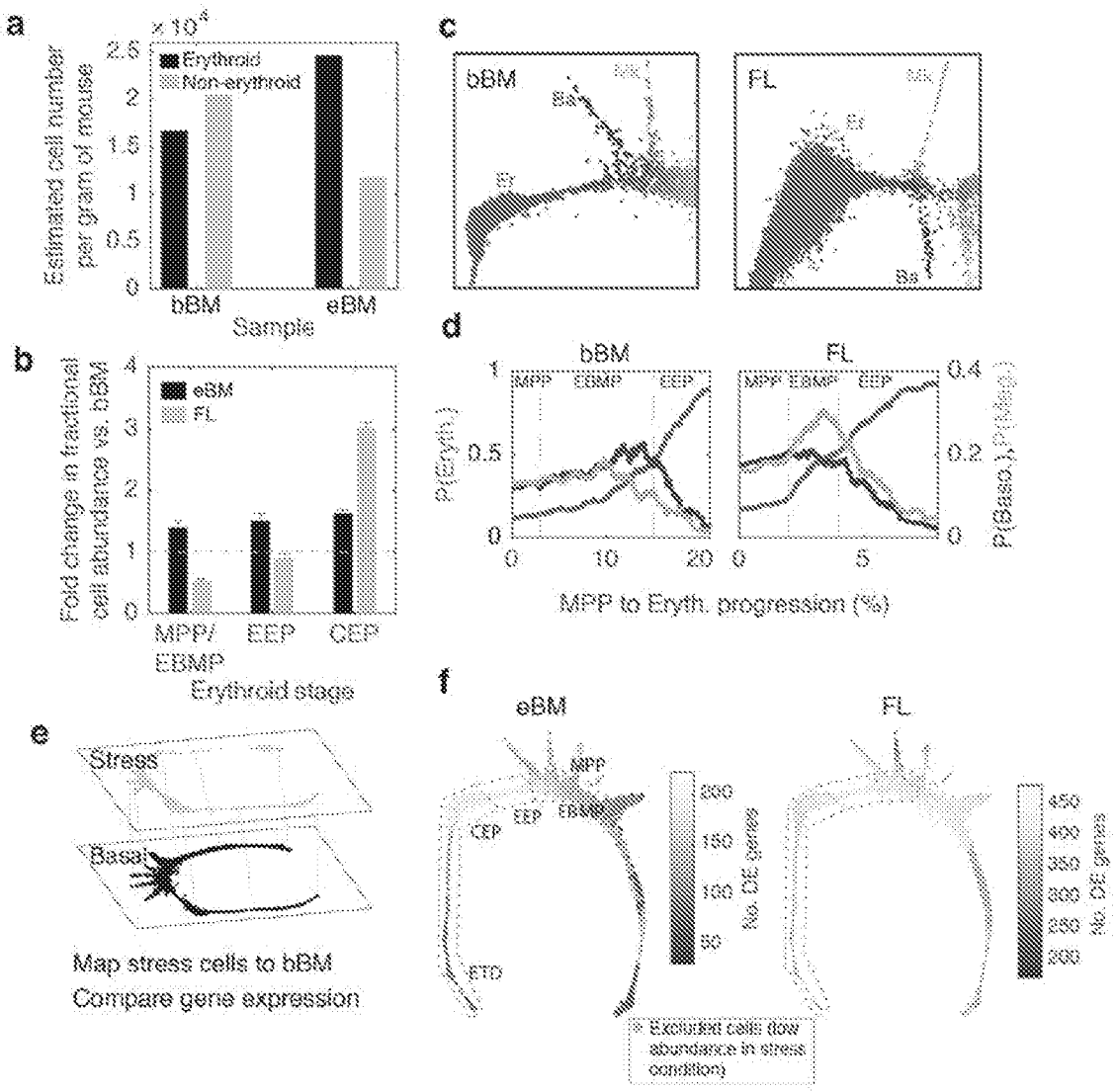


Figure 6 (cont.)

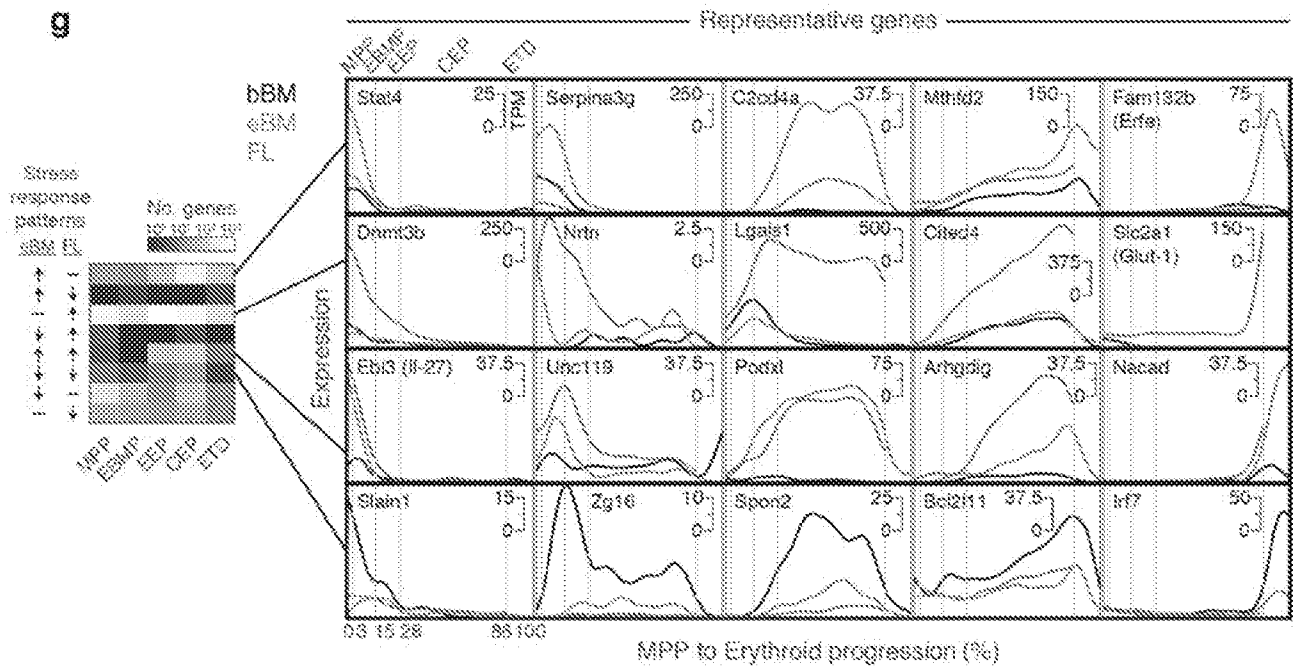

g
Representative genes
MPP
EBMP
BEP
CEP
ETO
bBM
eBM
FL
Stat4
Serpina3g
C2cd4a
Mthfd2
Fam132b (Erfe)
Dnmt3b
Nrtn
Lgals1
Cited4
Slc2a1 (Glut-1)
Ebi3 (Il-27)
Unc119
Podxl
Arhgdig
Nacad
Slain1
Zg16
Spon2
Bcl2l11
Irf7
Stress response patterns
No. genes
Expression
MPP to Erythroid progression (%)

Figure 7

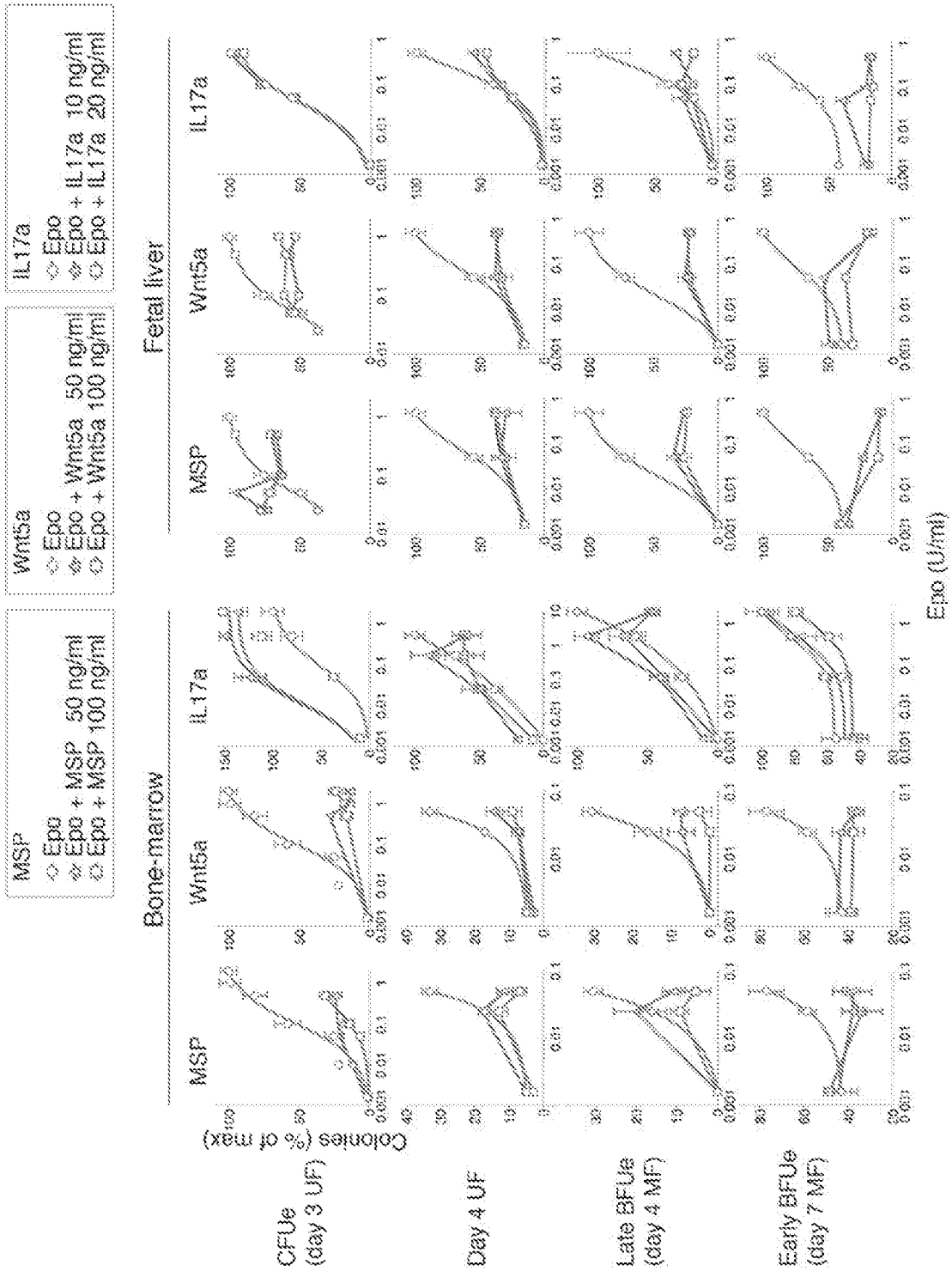

MSP
Epo
Epo + MSP 50 ng/ml
Epo + MSP 100 ng/ml
Wnt5a
Epo
Epo + Wnt5a 50 ng/ml
Epo + Wnt5a 100 ng/ml
IL17a
Epo
Epo + IL17a 10 ng/ml
Epo + IL17a 20 ng/ml
Bone-marrow
Fetal liver
MSP
Wnt5a
IL17a
Colonies (% of max)
CFUe (day 3 UF)
Day 4 UF
Late BFUe (day 4 MF)
Early BFUe (day 7 MF)
Epo (U/ml)

Figure 8

SEQ ID NO.	Accession ID	Protein name	Sequence
1	NM_001244937.2	MST1r	1 ggatcctcta gggtcccagc tcgcctcgat ggagctcctc ccgccgctgc ctcagtcctt 61 cctgttgctg ctgctgttgc ctgccaagcc cgcggcgggc gaggactggc agtgcccgcg 121 cacccctcac gcggcctctc gcgactttga cgtgaagtac gtggtgccca gcttctccgc 181 cggaggcctg gtacaggcca tggtgaccta cgaggggcgac agaaatgaga gtgctgtgtt 241 tgtagccata cgcaatcgcc tgcatgtgct tgggcctgac ctgaagtctg tccagaggcc 301 ggccacgggc cctgctggag acctggctg ccagacgtgt gcagcctgtg gcccaggacc 361 cacggccct cccggtgaca cagacacaaa ggtgctggtg ctggatcccg cgctgctgc 421 gctggtcagt tgtggctcca gcctgcaggg ccgtgcttc ctgcatgacc tagagcccca 481 agggacagcc gtgcatctgg cagcgccagc ctgcctcttc tcagcccacc atgaccggcc 541 cgatgactgc cccgactgtg tggccagccc atgtgggcac cgtgtaactg tggttgagca 601 agccaggccc tcctatttct acgtggcatc ctcactggac gcagccctgg ctgccagctt 661 cagcccacgc tcagtgtcta tcaggcgtct caaggctgac gcctcgggat tcgcaccggg 721 cttttgtggcg ttgtcagtgc tgcccaagca tcttgtctcc tacagtattg aatacgtgca 781 cagcttccac acgggagcct tcgtatactt cctgactgta cagcgggcca gcgtgacaga 841 tgatcctagt gcctgcaca cacgcctggc acggcttagc gccactgagc cagagttggg 901 tgactatcgg gagctggtcc tcgactgcag attgctcca aaacgcaggc gccgggggcc 961 cccagaaggc ggacagccct accctgtgct gcgggtggcc cactccgctc cagtgggtgc 1021 ccaacttgcc actgagctga gcatcgccga gggccaggaa gtactatttg gggtctttgt 1081 gactggcaag gatggtggtc ctggcgtggg ccccaactct gtcgtctgtg ccttcccgat 1141 tgacctgctg gacacactaa ttgatgaggg tgtggagcgc tgttgtgaat ccccagtcca 1201 tccaggcctc cggcgaggcc tcgacttctt ccagtcgccc agtttttgcc ccaacccgcc 1261 tggcctggaa gccctcagcc ccaacaccag ctgccgccac ttccctctgc tggtcagtag 1321 cagcttctca cgtgtggacc tattcaatgg gctgttggga ccagtacagg tcactgcatt 1381 gtatgtgaca cgccttgaca acgtcacagt ggcacacatg ggcacaatgg atgggcgtat 1441 cctgcaggtg gagctggtca ggtcactaaa ctacttgctg tatgtgtcca acttctcact 1501 gggtgacagt gggcagcccg tgcagcgtga tgtcagtcgt cttgtggacc acctactctt 1561 tgcctctggg gaccaggttt tccaggtacc tatccaaggc cctggctgcc gccactccct 1621 gacctgtggg cgttgcctaa gggcatggca ttttcatggc tgtggctggt gtgggaacat 1681 gtcggccag cagaaggagt gtcctggctc ctggcaacag gaccactgcc cacctaagct 1741 tactgagttc caccccaca gtggacctct aaggggcagt acaaggctga ccctgtgtgg 1801 ctccaacttc tacctcacc cttctggtct ggtgcctgag ggaacccatc aggtcactgt 1861 gggccaaagt ccctgcccgc cactgcccaa ggacagctca aaactcagac cagtgcccg 1921 gaaagacttt gtagaggagt ttgagtgtga actggagccc ttgggcaccc aggcagtggg 1981 gcctaccaac gtcagcctca ccgtgactaa catgccaccg ggcaagcact tccgggtaga 2041 cggcacctcc gtgctgagag gcttctcttt catggagcca gtgctgatag cagtgcaacc 2101 cctctttggc ccacgggcag gaggcacctg tctcactctt gaaggccaga gtctgtctgt 2161 aggcaccagc cgggctgtgc tggtcaatgg gactgagtgt ctgctagcac gggtcagtga 2221 ggggcagctt ttatgtgcca caccctgtgg ggccacgggtg gccagtgtcc ccttagcct 2281 gcaggtgggg ggtgcccagg tacctggttc ctggacctcc cagtacagag aagaccctgt

Figure 8 (cont.)

			2341 cgtgctaagc atcagcccca actgtggcta catcaactcc cacatcacca tctgtggcca 2401 gcatctaact tcagcatggc actagtgcct gtcattccat gacgggctta gggcagtgga 2461 aagcagtgtg gagaggcagc ttccagagca gcagctgtgc cgcttcctg aatatgtggt 2521 ccgagacccc cagggatggg tggcagggaa tctgagtgcc cgagggggatg gagctgctgg 2581 cttacactg cctggcttcc gcttcctacc cccacccat ccacccagtg ccaacctagt 2641 tccactgaag cctgaggagc atgccattaa gtttgaggtc tgcgtagatg gtgaatgtca 2701 tatcctgggt agagtgggtgc ggccagggcc agatgggggtc ccacagagca cgctccttgg 2761 tatcctgctg ccttgctgcc tgcttgtggc tgcactggcg actgcactgg tcttcagcta 2821 ctggtggcgg aggaagcagc tagttcttcc tcccaacctg aatgacctgg catccctgga 2881 ccagactgct ggagccacac ccctgcctat tctgtactcg ggctctgact acagaagtgg 2941 cctgcactc cctgccattg atggtctgga ttccaccact tgtgtccatg gagcatcctt 3001 ctccgatagt gaagatgaat cctgtgtgcc actgctgcgg aaagagtcca tccagctaag 3061 ggacctggac tctgcgctct tggctgaggt caaggatgtg ctgattcccc atgagcggct 3121 ggtcacccac agtgaccgag tcattggcaa aggccacttt ggagttgtct accacggaga 3181 atacatagac caggcccaga atcgaatcca atgtgccatc aagtcactaa gtcgcatcac 3241 agagatgcag caggtggagg cctcctgccg agaggggctg ctcatgcgtg gcctgaacca 3301 cccgaatgtg ctggctctca ttggtatcat gttgccacct gaggcctgc cccatgtgct 3361 gctgccctat atgtgccacg gtgacctgct ccagttcatc cgctcacctc agcggaaccc 3421 caccgtgaag gacctcatca gctttggcct gcaggtagcc cgcggcatgg agtacctggc 3481 agagcagaag ttgtgcaca gggacctggc tgcgcggaac tgcatgctgg acgagtcatt 3541 cacagtcaag gtggctgact ttggtttggc ccgcgacatc ctggacaggg agtactatag 3601 tgttcaacag catcgccacg ctcgcctacc tgtgaagtgg atggcgctgg agagcctgca 3661 gacctataga ttaccacca agtctgatgt gtggtcattt ggtgtgctgc tgtgggaact 3721 gctgacacgg ggtgccccac cataccgcca cattgaccct ttgacctta cccacttcct 3781 ggcccagggt cggcgcctgc cccagcctga gtattgccct gattctctgt accaagtgat 3841 gcagcaatgc tgggaggcag acccagcagt gcgaccacc ttcagagtac tagtgggga 3901 ggtggagcag atagtgtctg cactgcttgg ggaccattat gtgcagctgc cagcaaccta 3961 catgaacttg ggccccagca cctcgcatga gatgaatgtg cgtccagaac agccgcagtt 4021 ctcacccatg ccagggaatg tacgccggcc ccggccactc tcagagcctc ctcggcccac 4081 ttgacttagt tcttgggctg gacctgctta gctgccttga gctaacccca agctgcctct 4141 gggccatgcc aggccagagg gcagtgccc tccacctgt tcctgccctt taactttcag 4201 aggcaatagg taaatgggc ccattaggtc cctcactcca cagatgagc cagtgagggc 4261 agtcctgcaa catgtattta tggagtgcct gctgtggacc ctgtcttctg ggcacagtgg 4321 actcagcagt gaccacacca acactgaccc ttgaaccaat aaaggaacaa atgactatta 4381 aagcacaaaa aaaaaaaaaa aa	2	NP_001231866.1 MSTIr	1 mellpplpgs fllillpak paagedwqcp rtpyaasrdf dvkyvvpsfs agglvqamvt 61 yegdrnesav fvairnrlhv lgpdlksvgs latgpagdpg cqtcaacgpg phgppgdtdt 121 kvividpalp alvscgssiq grcflhdlep qgtavhlaap aclfsahhnr pddcpdcvas 181 plgtrvtvve qggasyfyva ssldaavaas fsprsvsirr lkadasgfap gfvalsvipk 241 hlvsysieyv hsfhtgafvy fltvqpasvt ddpsalhtrl arlsatepei gdyrelvldc 301 rfapkrrrrg apeggqpypv lrvahsapvg aqlatelsia eggevlfgvf vtgkdqgpsv 361 qpnsvvcafp idlldtlide gverccespv hpglrrgldf fqspsfcpnp pglealspnt

Figure 8 (cont.)

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421 scrhfpllvs ssfsrvdlfn gllgpvqvta lyvtrldnvt vahmgtmdgr ilqvelvrsi
481 nyllyvsnfs lgdsgppvqr dvsrlgdhll fasgdqvfqv piggpgcrhf ltcgrclraw
541 hfmgcgwcgn mcgqqkecpg swqqdhcppk ltefhphsgp lrgstrltlc gsnfylhpsg
601 ivpegthqvt vgqspcrpip kdssklrpvp rkdfveefec eleplgtqav gptnvsltvt
661 nmppgkhfrv dgtsvlrgfs fmepvliavq pifgpraggt cltieggsls vgtsravivn
721 gteciarvs eggllcatpp gatvasvpls lqvggaqvpg swtfqyredp vvlsispncg
781 yinshiticg qhltsawhiv lsfhdglrav esrcerglpe gqlcripeyv vrdpqgwvag
841 nlsargdgaa gftlpgfrfl ppphppsanl vplkpeehai kfevcvdgec hilgrvvrpg
901 pdgvpqstll gillpllllv aalatalvfs ywwrrkqlvl ppnlndlasl dqtagatplp
961 ilysgsdyrs glalpaidgl dsttcvhgas fsdsedescv pllrkesigl rdldsaliae
1021 vkdvlipher vvthsdrvig kghfgvvyhg eyidgaqnri qcaikslsri temgqveafl
1081 regllmrgln hpnvlaligi mlppeglphv llpymchgdl lqfirspqrn ptvkdlisfg
1141 lqvargmeyl aeqkfvhrdl aarncmldes ftvkvadfgl ardiidreyy svqqhrharl
1201 pvkwmalesl qtyrfttksd vwsfgvllwe lltrgappyr hidpfditthf laggrrlpqp
1261 eycpdslyqv mqqcweadpa vrptfrvlvg eveqivsall gdhyvqlpat ymnlgpstsh
1321 emnvrpeqpq fspmpgnvrr prplseppqp t
```

Figure 9

SEQ ID NO.	Accession ID	Protein name	Sequence
3	NM_020998.3	Msp	1 cagcaggccc tgagctgacg tgtggagcca gagccaccca atccgtagg gacaggttc 61 acaacttccc ggatgggct gtggtgggtc acagtgcagc ctccagccag aaggatgggg 121 tggctcccac tcctgctgct tctgactcaa tgcttagggg tccctgggca gcgctcgcca 181 ttgaatgact tccaagtgct ccggggcaca gagctacagc acctgctaca tgcggtggtg 241 cccgggcctt ggcaggagga tgtggcagat gctgaagagt gtgctggtcg ctgtgggccc 301 ttaatggact gccgggcctt ccactacaac gtgagcagcc atggttgcca actgctgcca 361 tggactcaac actcgcccca cacgaggctg cggcgttctg ggcgctgtga cctcttccag 421 aagaaagact acgtacggac ctgcatcatg aacaatgggg ttgggtaccg gggcaccatg 481 gccacgaccg tgggtggcct gccctgccag gcttggagcc acaagttccc aaatgatcac 541 aagtacacgc ccactcccg gaatggcctg gaagagaact tctgccgtaa ccctgatggc 601 gaccccggag gtccttggtg ctacacaaca gaccctgctg tgcgcttcca gagctgcggc 661 atcaaatcct gccgggaggc cgcgtgtgtc tggtgcaatg gcgaggaata ccgcgggcgg 721 gtagaccgca cggagtcagg gcgcgagtgc cagcgctggg atcttcagca cccgcaccag 781 caccccttcg agccgggcaa gttcctcgac caaggtctgg acgacaacta ttgccggaat 841 cctgacggct ccgagcggcc atggtgctac actacggatc cgcagatcga gcgagagttc 901 tgtgacctcc cccgctgcgg gtccgaggca cagccccgcc aagagcccac aactgtcagc 961 tgcttccgcg ggaagggtga gggctaccgg ggcacagcca ataccaccac tgcgggcgta 1021 ccttgccagc gttggacgc gcaaatcccg catcagcacc gatttacgcc agaaaaatac 1081 gcgtgcaaag accttcggga gaacttctgc cggaaccccg acggctcaga ggccgccctgg 1141 tgcttcacac tgcggccccg catgcgcgcg gcctttgct accagatccg gcgttgtaca 1201 gacgacgtgc ggccccagga ctgctaccac ggcgcagggg agcagtaccg cggcacggtc 1261 agcaagaccc gcaagggtgt ccagtgccag cgctggtccg ctgagacgcc gcacaagccg 1321 cagttcacgt ttacctccga accgcatgca caactggagg agaacttctg ccggaaccca 1381 gatgggata gccatgggcc ctggtgctac acgatggacc caaggacccc attcgactac 1441 tgtgccctgc gacgctgcgc tgatgaccag ccgccatcaa tcctggaccc cccagaccag 1501 gtgcagttg agaagtgtgg caagagggtg gatcggctgg atcagcggcg ttccaagcta 1561 cgcgtggttg gggccatcc gggcaactca ccctggacag tcagcttgcg gaatcggcag 1621 ggccagcatt tctgcggggg gtctctagtg aaggagcagt ggatactgac tgccggcag 1681 tgcttctcct cctgccatat gcctctcacg ggctatgagg tatggttggg caccctgttc 1741 cagaacccac agcatggaga gccaagccta cagcgggtcc cagtagccaa gatggtgtgt 1801 gggccctcag gctcccagct tgtcctgctc aagctggaga gatctgtgac cctgaaccag

Figure 9 (cont.)

4	NP_066278.3	Msp	1861 cgtgtggccc tgatctgcct gccccctgaa tggtatgtgg tgcctccagg gaccaagtgt 1921 gagattgcag gctgggggtga gaccaaaggt acgggtaatg acacagtcct aaatgtggcc 1981 ttgctgaatg tcatctccaa ccaggagtgt aacatcaagc accgaggacg tgtgcgggag 2041 agtgagatgt gcactgaggg actgttggcc cctgtgggag cctgtgaggg tgactacggg 2101 ggcccacttg cctgctttac ccacaactgc tgggtcctgg aaggaattat aatcccaac 2161 cgagtatgcg caaggtcccg ctggccagct gtcttcacgc gtgtctctgt gtttgtggac 2221 tggattcaca aggtcatgag actgggttag gcccagcctt gatgccatat gccttgggga 2281 ggacaaaact tcttgtcaga cataaagcca tgttcctct ttatgcctgt aaaaaaaaa 2341 aaaaaaaa 1 mglwwvtvgp parmgwlpl lllltqclgv pgqrsplndf qvlrgteiqh llhavvpgpw 61 qedvadaeec agrcgplmdc rafhynvssh gcqilpwtqh sphtrlrrsg rcdlfqkkdy 121 vrtcimngv gyrgtmattv gglpcqawsh kfpndhkytp tlrngleenf crnpdqdpgg 181 pwcyttdpav rfgscgiksc reaacvwcng eeyrgavdrt esgrecqrwd lqhphqhpfe 241 pgkfldqgld dnycrnpdgs erpwcyttqp qierefcdlp rcgseaqprq eattvscfrg 301 kgegyrgtan tttagvpcqr wdaqiphqhr ftpekyackd lrenfcrnpd gseapwcfti 361 rpgmraafcy qirictddvr pgdcyhgage qyrgtvsktr kgvqcqrwsa etphkpqftf 421 tsephaglee nfcrnpdgds hgpwcytmdp rtpfdycalr rcaddqppsi ldpdqvqfe 481 kcgkrvdrld qrrsklrvvg ghpgnspwtv sirnrqgghf cggslvkeqw iltarqcfss 541 chmpltgyev wlgtlfqnpq hgepslqrvp vakmvcgpsg sqlvlkier svtlnqrval 601 icippewyvv ppgtkceiag wgetkgtgnd tvlnvallnv isnqecnikh rgrvresemc 661 tegliapvga cegdyggpla cfthncwvle gilipnrvca rsrwpavftr vsvfvcwihk 721 vmrlg

Figure 10

SEQ ID NO.	Accession ID	Protein name	Sequence
5	NM_001005861.2	RYK	1 cggctcgggg ctgtgagcgg ctcggggccg ggggtgggcg gcggtgcggc gggcggccga 61 cgctcctctt cggcggcggc ggcggcggcc atgcgtgggg cggcgcggct ggggcggccg 121 ggccggagtt gcctcccggg ggcccgcggc ctgagggccc cgccgccgcc gccgctgctg 181 cttctgcttg cgctgttgcc gctgctgccc gcgcctggcg ctgccgccgc ccccgccccg 241 cggcccccgg agctgcagtc ggcttccgcg gggcccagcg tgagtctcta cctgagcgag 301 gacgaggtgc gccggctgat cggtcttgat gcagaacttt attatgtgag aaatgacctt 361 attagtcact acgctctatc ctttagtctg ttagtaccca gtgagacaaa tttcctgcac 421 ttcacctggc atgcgaagtc caaggttgaa tataagctgg gattccaagt ggacaatgtt 481 ttggcaatgg atatgcccca ggtcaacatt tctgttcagg gggaagttcc acgcacttta 541 tcagtgtttc gggtagagct ttcctgtact ggcaaagtag attctgaagt tatgatacta 601 atgcagctca acttgacagt aaattcttca aaaaatttta ccgtcttaaa ttttaaacga 661 aggaaaatgt gctacaaaaa acttgaagaa gtaaaaactt cagccttgga caaaaacact 721 agcagaacta tttatgatcc tgtacatgca gctccaacca cttctacgcg tgtgttttat 781 attagtgtag gggtttgttg tgcagtaata tttctcgtag caataatatt agctgttttg 841 caccttcata gtatgaaaag gattgaactg gatgacagca ttagtgccag cagtagttcc 901 caagggctgt ctcagccatc cacccagacg actcagtatc tgagagcaga cacgcccaac 961 aatgcaactc ctatcaccag ctccttaggt tatcctacct tgcggataga gaagaacgac 1021 ttgagaagtg tcactctttt ggaggccaaa ggcaaggtga aggatatagc aatatccaga 1081 gagaggataa ctctaaaaga tgtactccaa gaaggtactt ttgggcgtat tttccatggg 1141 attttaatag atgaaaaaga tccaaataaa gaaaaacaag catttgtcaa aacagttaaa 1201 gatcaagctt ctgaaattca ggtgacaatg atgctcactg aaagttgtaa gctgcgaggt 1261 cttcatcaca gaaatcttct tcctattact catgtgtgta tagaagaagg agaaaagccc 1321 atggtgatat tgccttacat gaattggggg aatcttaaat tgtttttacg acagtgcaag 1381 ttagtagagg ccaataatcc acaggcaatt tctcagcaag acctggtaca catggctatt 1441 cagattgcct gtggaatgag ctacctggcc agaagggaag tcatccacaa agacctggct 1501 gccaggaact gtgtcattga tgacacactt caagttaaga tcacagacaa tgccctctcc 1561 agagacttgt tccccatgga ctatcactgt ctgggggaca atgaaaacag gccagttcgt 1621 tggatggctc ttgaaagtct ggttaataac gagttctcta gcgctagtga tgtgtgggcc 1681 tttggagtga cgctgtggga actcatgact ctgggccaga ctccctacgt ggacattgac 1741 cccttcgaga tggccgcata cctgaaagat ggttaccgaa tagcccagcc aatcaactgt 1801 cctgatgaat tatttgctgt gatggcctgt tgctgggcct tagatccaga ggagaggccc

Figure 10 (cont.)

		1861 aagtttcagc agctggtaca gtgcctaaca gagtttcatg cagccctggg ggcctacgtc 1921 tgactcctct ccaatcccac accatcagga agaaggtgcc tgtcgtggct cacttgaagc 1981 ctgtcaggga tgctttgtat ctaacacaac gccaacagaa gcacatttgt ctccagaac 2041 accgtgcctt agaaatgctt tagaatctga actttttaag acagacttaa taatgtggca 2101 tatttctag atatcacttt tattaggttg aactgaaagg gttttgtaa attttttggc 2161 caaaatttct taaaacatac ttactttgga ctagggtac attcttacaa aataaataa 2221 cagttttaa aattgtttag acacagatat ttggaattag ctatcttagt gccaactgct 2281 tttatttct ttacttcatc aaggtgatgt aagtgactca ctttaaagt ttttttagtg 2341 ttatttttta tcactactct gggaaatggt ttgtcttcaa gatgcaatac tttcttagt 2401 aaaggaaaaa cagcataaaa agatacctgg tctgccttgt acaagaaaag gcaatattag 2461 aggaagaaaa ttaaagaaa agctagagga aaaaaaatt ttttaaaaa tacttattag 2521 aagcaaactg ccctgcatg gaaaactgtt tatttttttc agtgaaaagg aattctgctt 2581 tcgtgttttt gggaaagcag gaactgagtt cattacatct ttaatttggc agaaattagc 2641 cttctgtga accagatgtg gtttgggga gatctgtagt aaacaatggt gattttattt 2701 attttactc tctggaaaag gagataatac aattccagaa agtgaactca tatttctaag 2761 gttaagattc cctttattg cacctagaat agtgctatgc acagagcggg tgcttgagtt 2821 gttgtcgttt tttgtttgtt tttaaatgt aaactggtaa atttgtgct tatcttcaag 2881 gctggcttaa gtataaaatt gttttttaaa cacttgaaaa attaaaggat ttgttttata 2941 tt		
6	NP_001005861.1	RYK	1 mrgaarlgrp grsclpgarg lrappppll lllallpllp apgaaaapap rppelgsasa 61 gpsvslylse devrrligld aelyyvrndl ishyalsfsl lvpsetnflh ftwhakskve 121 yklgfqvdnv lamdmpqvni svqgevprtl svfrvelsct gkvdsevmil mqlnltvnss 181 knftvlnfkr rkmcykklee vktsaldknt srtiydpvha apttstrvfy isvgvccavi 241 flvaillavl hlhsmkriel qdsisassss qglsqpstgt tgylradtpn natpitsslg 301 yptlrieknd lrsvtlleak gkvkdiaisr eritlkdvlq egtfgrifhg ilidekdpnk 361 ekgafvktvk dgaseiqvtm mltescklrg lhhrnllpit hvcieegekp mvilpymnwg 421 nlklflrgck lveanrpgai sqqdlvhmai qiacgmsyla rrevihkdla arncviddti 481 qvkitdnals rdlfpmdyhc lgdnenrpvr wmaleslvnn efssasdvwa fgvtlwelmt 541 lggtpyvdid pfemaaylkd gyriaqpinc pdeifavmac cwaldpeerp kfqqlvqclt 601 efhaalgayv

Figure 11

SEQ ID NO.	Accession ID	Protein name	Sequence
7	NM_001256105.1	WNT5a	1 gctccactcg cctccgtgct cctctcgccc atggaattaa ttctggctcc acttgttgct 61 cggcccagaa gtccattgga atattaagcc caggagttgc tttgtggatg gctggaagtg 121 caatgtcttc caagttcttc ctagtggctt tggccatatt tttctcctte gcccaggttg 181 taattgaagc caattcttgg tggtcgctag gtatgaataa ccctgttcag atgtcagaag 241 tatatattat aggagcacag cctctctgca gccaactggc aggactttct caaggacaga 301 agaaactgtg ccacttgtat caggaccaca tgcagtacat cggagaaggc gcgaagacag 361 gcatcaaaga atgccagtat caattccgac atcgaaggtg gaactgcagc actgtggata 421 acacctctgt tttggcagg gtgatgcaga taggcagccg cgagacggcc ttcacatacg 481 cggtgagcgc agcagggctg gtgaacgcca tgagccgggc gtgccgcgag ggcgagctgt 541 ccacctgcgg ctgcagccgc gccgccgccc ccaaggacct gccgcgggac tggctctggg 601 gcggctgcgg cgacaacatc gactatggct accgctttgc caaggagttc gtggacgccc 661 gcgagcgggg gcgcatccac gccaagggct cctacgagag tgctcgcatc ctcatgaacc 721 tgcacaacaa cgaggccggc cgcaggacgg tgtacaacct ggctgatgtg gcctgcaagt 781 gccatgggct gtccggctca tgtagcctga agacatgctg gctgcagctg gcagacttcc 841 gcaaggtggg tgatgccctg aaggagaagt acgacaagcc ggcggccatg cggctcaaca 901 gccggggcaa gttggtacag gtcaacagcc gcttcaactc gcccaccaca caagacctgg 961 tctacatcga cccagccct gactactgcg tgcgcaatga gagcaccggc tcgctgggca 1021 cgcagggccg cctgtgcaac aagacgtcgg agggcatgga tggctgcgag ctcatgtgct 1081 gcggccgtgg ctacgaccag ttcaagaccg tgcagacgga gcgctgccac tgcaagttcc 1141 actggtgctg ctacgtcaag tgcaagaagt gcacggagat cgtggaccag tttgtgtgca 1201 agtagtgggt gccacccagc actcagcccc gctcccagga cccgcttatt tatagaaagt 1261 acagtgattc tggttttgg ttttagaaa tatttttat ttttccccaa gaattgcaac 1321 cggaaccatt ttttttcctg ttaccatcta agaactctgt ggtttattat taatattata 1381 attattattt ggcaataatg gggtgtggaa ccaagaaaaa tatttatttt gtggatcttt 1441 gaaaaggtaa tacaagactt ctttgatag tatagaatga aggggaaata acacataccc 1501 taacttagct gtgtggacat ggtacacatc cagaaggtaa agaaatacat tttctttttc 1561 tcaaatatgc catcatatgg gatgggtagg ttccagttga aagagggtgg tagaaatcta 1621 ttcacaattc agcttctatg accaaaatga gttgtaaatt ctctggtgca agataaaagg 1681 tcttgggaaa acaaaacaaa acaaaacaaa cctccttcc ccagcagggc tgctagcttg 1741 ctttctgcat ttttcaaaatg ataatttaca atggaaggac aagaatgtca tattctcaag 1801 gaaaaaaggt atatcacatg tctcatttct ctcaaatatt ccatttgcag acagaccgtc

Figure 11 (cont.)

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1861 atattctaat agctcatgaa atttgggcag cagggaggaa agtcccaga aattaaaaaa
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1981 tgtaaaaaga tcccaaatg attctggaca ctagattttt ttgttgggg aggttggctt
2041 gaacataaat gaaaatatcc tgttatattc ttagggatac ttggttagta aattataata
2101 gtaaaaataa tacatgaatc ccatticacag gttcticagcc caagcaacaa ggtaattgcg
2161 tgccattcag cactgcacca gagcagacaa cctatttgag gaaaacagt gaaatccacc
2221 ttcctcttca cactgagccc tctctgattc ctccgtgttg tgatgtgatg ctggccacgt
2281 ttccaaacgg cagctccact gggtcccctt tggttgtagg acaggaaatg aaacattagg
2341 agctctgctt ggaaaacagt tcactactta gggatttttg tttcctaaaa ctttattttt
2401 gaggagcagt agtttctat gtttaatga cagaacttgg ctaatggaat tcacagaggt
2461 gttgcagcgt atcactgtta tgatcctgtg tttagattat ccactcatgc ttctcctatt
2521 gtactgcagg tgtaccttaa aactgttccc agtgtactg aacagttgca tttataaggg
2581 gggaaatgtg gtttaatggt gcctgatatc tcaaagtctt ttgtacataa catatatata
2641 tatatacata tatataaata taaataataa tatatctcat tgcagccagt gatttagatt
2701 tacagtttac tctgggttta tttctctgtc tagagcattg ttgtccttca ctgcagtcca
2761 gttgggatta ttccaaaagt tttttgagtc ttgagcttgg gctgtgccc tgctgtgatc
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2941 tccatttctg ttcatttgt ggagagggca ttacttgttc gttatagaca tggacgttaa
3001 gagatattca aaactcagaa gcatcagcaa tgttctcttt ttcttagttc attctgcaga
3061 atggaaaccc atgcctatta gaaatgacag tacttattaa ttgagtccct aaggaatatt
3121 cagccacta catagatagc ttttttttt ttttttttaa taaggacacc tctttccaaa
3181 cagtgccatc aaatatgttc ttatctcaga cttacgttgt tttaaaagtt tggaaagata
3241 cacatctttc atacccccct taggcaggtt ggctttcata tcacctcagc caactgtggc
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3541 aatatttctac atgtcattca gatattatgt atatctccta gcctttattc tgtactttta
3601 atgtacatat ttctgtcttg cgtgatttgt atatttcact ggtttaaaaa acaaaacatcg
3661 aaaggcttat gccaaatgga agatagaata taaaataaaa cgttacttgt atattggtaa
3721 gtggtttcaa ttgtccttca gataatttcat gtggagaatc ttggagaaac catgacggat
3781 agtttaggat gactacatgt caaagtaata aaagagtggt gaatttacc aaaaccaagc
3841 tatttggaag cttcaaaagg ttttctatatg taatggaaca aaaggggaat tctctttccc
3901 tatatatgtt ccttacaaaa aaaaaaaaaa aagaaatcaa gcagatggct taaagctggt
3961 tataggattg ctcacattct tttagcatta tgcatgtaac ttaattgttt tagagcgtgt
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Figure 11 (cont.)

		4021 tgctgttgta acatcccaga gaagaatgaa aaggcacatg ctttatccg tgaccagatt 4081 tttagtccaa aaaaatgtat ttttttgtgt gtttaccact gcaactattg cacctctcta 4141 tttgaattta ctgtggacca tgtgtggtgt ctctatgccc tttgaaagca gttttataa 4201 aaagaaagcc cgggtctgca gagaatgaaa actggttgga aactaaaggt tcattgtgtt 4261 aagtgcaatt aatacaagtt attgtgcttt tcaaaaatgt acacggaaat ctggacagtg 4321 ctccacagat tgatacatta gccttttgctt tttctctttc cggataacct tgtaacatat 4381 tgaaacctttt taaggatgcc aagaatgcat tattccacaa aaaaacagca gaccaacata 4441 tagagtgttt aaaatagcat ttctgggcaa attcaaactc ttgtggttct aggactcaca 4501 tctgtttcag ttttcctca gttgtatatt gaccagtgtt ctttattgca aaaacatata 4561 cccgatttag cagtgtcagc gtatttttttc ttctcatcct ggagcgtatt caagatcttc 4621 ccaatacaag aaaattaata aaaaatttat atataggcag cagcaaaaga gccatgttca 4681 aaatagtcat tatgggctca aatagaaaga agacttttaa gttttaatcc agtttatctg 4741 ttgagttctg tgagctactg acctcctgag actggcactg tgtaagtttt agttgcctac 4801 cctagctctt ttctcgtaca atttgtccaa taccaagttt caatttgttt ttacaaaaca 4861 ttattcaagc cactagaatt atcaaatatg acgctatagc agagtaaata ctctgaataa 4921 gagaccggta ctagctaact ccaagagatc gttagcagca tcagtccaca aacacttagt 4981 ggcccacaat atatagagag atagaaaagg tagttataac ttgaagcatg tatttaatgc 5041 aaataggcac gaaggcacag gtctaaaata ctacattgtc actgtaagct atactttaa 5101 aatatttatt ttttttaaag tatttttctag tctttttctct ctctgtggaa tggtgaaaga 5161 gagatgccgt gttttgaaag taagatgatg aaatgaattt ttaattcaag aaacattcag 5221 aaacatagga attaaaactt agagaaatga tctaatttcc ctgttcacac aaactttaca 5281 ctttaatctg atgattggat atttatattt agtgaaacat catcttgtta gctaacttta 5341 aaaaatggat gtagaatgat taaaggttgg tatgatttct tttaatgta tcagtttgaa 5401 cctagaatat tgaattaaaa tgctgtctca gtatttttaa agcaaaaaag gaatggagga 5461 aaattgcatc ttagaccatt tttatatgca gtgtacaatt tgctgggcta gaaatgagat 5521 aaagattatt tatttttgtt catatcttgt actttttctat taaaatcatt ttatgaaatc 5581 caaaaaaaaa aaaaaaaaa
8	NP_001243034.1	WNT5a	1 magsamsskf flvalaiffs fagvvieans wwslgmmnpv qmsevyiga qplcsqlagi 61 sqgqkkichl ygdhmqyige gaktgikecq yqfrhrrwnc stvdntsvfg rvmqigsret 121 aftyavsaag vvnamsracr egelstcgcs raarpkdlpr dwlwggcgdn idygyrfake 181 fvdarereri hakgsyesar ilmnlhnnea grrtvynlad vackchgvsg scslktcwlq 241 iadfrkvgda lkekydsaaa mrlnsrgklv qvnsrfnspt tgdlvviqps pdycvrnest 301 gslgtqgrlc nktsegmdgc elmccgrgyd qfktvqterc hckfhwccyv kckkcteivd 361 qfvck

Figure 12

SEQ ID NO.	Accession ID	Protein name	Sequence
9	NM_014339.6	IL-17ra	1 ctgggcccgg gctggaagcc ggaagcgagc aaagtggagc cgactcgaac tccaccgcgg 61 aaaagaaagc ctcagaacgt tcgttcgctg cgtccccagc cggggccgag ccctccgcga 121 cgccagccgg gccatggggg ccgcacgcag cccgccgtcc gctgtcccgg ggcccctgct 181 ggggctgctc ctgctgctcc tgggcgtgct ggccccgggt ggcgcctccc tgcgactcct 241 ggaccaccgg gcgctggtct gctcccagcc ggggctaaac tgcacggtca agaatagtac 301 ctgcctggat gacagctgga ttcaccctcg aaacctgacc ccctcctccc caaaggacct 361 gcagatccag ctgcactttg cccacaccca acaaggagac ctgttccccg tggctcacat 421 cgaatggaca ctgcagacag acgccagcat cctgtacctc gagggtgcag agttatctgt 481 cctgcagctg aacaccaatg aacgtttgtg cgtcaggttt gagtttctgt ccaaactgag 541 gcatcaccac aggcggtggc gttttacctt cagccacttt gtggttgacc ctgaccagga 601 atatgaggtg accgttcacc acctgcccaa gcccatccct gatggggacc caaaccacca 661 gtccaagaat ttccttgtgc ctgactgtga gcacgccagg atgaaggtaa ccacgccatg 721 catgagctca ggcagcctgt gggaccccaa catcaccgtg gagaccctgg aggcccacca 781 gctgcgtgtg agcttcaccc tgtggaacga atctacccat taccagatcc tgctgaccag 841 ttttccgcac atggagaacc acagttgctt tgagcacatg caccacatac ctgcgcccag 901 accagaagag ttccaccagc gatccaacgt cacactcact ctacgcaacc ttaaagggtg 961 ctgtcgccac caagtgcaga tccagccctt cttcagcagc tgcctcaatg actgcctcag 1021 acactccgcg actgtttcct gcccagaaat gccagacact ccagaaccaa ttccggacta 1081 catgcccctg tgggtgtact ggttcatcac gggcatctcc atcctgctgg tgggctccgt 1141 catcctgctc atcgtctgca tgacctggag gctagctggg cctggaagtg aaaaatacag 1201 tgatgacacc aaatacaccg atggcctgcc tgcggctgac ctgatccccc caccgctgaa 1261 gcccaggaag gtctggatca tctactcagc cgaccacccc ctctacgtgg acgtggtcct 1321 gaaattcgcc cagttcctgc tcaccgcctg cggcacggaa gtggccctgg acctgctgga 1381 agagcaggcc atctcggagg caggagtcat gacctgggtg ggccgtcaga agcaggagat 1441 ggtggagagc aactctaaga tcatcgtcct gtgctcccgc ggcacgcgcg ccaagtggca 1501 ggcgctcctg ggccgggggg cgcctgtgcg gctgcgctgc gaccacggaa agcccgtggg 1561 ggacctgttc actgcagcca tgaacatgat cctcccggac ttcaagaggc cagcctgctt 1621 cggcacctac gtagtctgct acttcagcga ggtcagctgt gacggcgacg tccccgacct 1681 gttcggcgcg gcgccgcggt acccgctcat ggacaggttc gaggaggtgt acttccgcat 1741 ccaggacctg gagatgttcc agccgggccg catgcaccgc gtaggggagc tgtcgggga 1801 caactacctg cggagcccgg gcggcaggca gctccgcgcc gccctggaca ggttccggga

Figure 12 (cont.)

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1981 catcgtgaag cgggcgcccc tggtgcgcga gcctggctcc caggcctgcc tggccataga
2041 cccgctggtc ggggaggaag gaggagcagc agtggcaaag ctggaacctc acctgcagcc
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Figure 12 (cont.)

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4561 gtccttaact atatttccaa cctccagtga ggaggagaag attcggaaat gtgacaggag
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Figure 12 (cont.)

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6181 cactgcagcc tcgacctccc aggctcaagc catctttcca cctcagcctg ccagtggcta
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7201 ctccatgccc catcgcctct cccagatcct agcactgggt ccacactctc gccctgtcca
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7741 tggtgcacac tggctttct tcctgactgc aatgtggcat tgtgccagct acctcctctt
7801 tctcggcctc aggaaaatgg agagaaagca gccctgaagg tggctgtgac gagggaaggg
7861 gcagagggcc tgacagtcaa ccacgcgcta tattttcctg ttcttcctta gggcaagaac
7921 tgcatgggcca gactcaggca aggcctaggt gtgggctggg cattgcctac acgtgaagag
7981 atcactccgc gtcctactg cacctgtcac aaagtgcctt ctgatatgcc tggcaaacca
8041 aaatcggtga gcgccagctt gcttccctag aagacatttc taaatattca taacatgctt
8101 gctcaaatca atcacttat tttacatccg ctccagggag aaatgaagac atggtcctac
8161 gttgttctgt aattatttc tatgtaaatt ttgttccttg ttacaattat atatgtctta
8221 gggaaagga ccatttcaca tgtgtcacct catgtgattc tcaccacagc cctgtgattg
8281 ctcctgtttt ataaataatg acatagttcc agttgatggc caaagccaca gctaacgaga
```

Figure 12 (cont.)

		8341 ggcagagaga gctcaggctc ccaggagctt ccactctcag accttgcctc ccgggctgcc 8401 ctgagtgaaa cgcctgctta gcatttggca cagccagaag cagcaagcta gggtcacaac 8461 acagagaggg gctgtgtaat actggctgcc tctgtgctaa gaaaaaaaaa aaatcactgt 8521 gtgtttgttt atttggtgtc aggcccagtg ttcttgctta gacttaatac taccttcat 8581 gttaaaataa aaccaaacaa aaacccat
10	NP_001276834.1	IL-17ra	1 mgaarsppsa vpgpllglll lllgvlapgg aslrlldhra lvcsgpginc tvknstcldd 61 swihprnltp sspkdlqlql hfahtqqgdl fpvahiewtl qtdasilyle gaelsvlqln 121 tnerlcvrfe flsklrhhhr rwrftfshfv vdpdqeyevt vhhlpkpipd gdpnhqsknf 181 ivpdceharm kvttpcmssg slwdpnitve tleahqlrvs ftlwnesthy qillsfphm 241 enhscfehmh hipaprpeef hqrsnvtltl rnlkgccrhq vqigpffssc lndclrhsat 301 vscpempdtp epipgpggsek ysddtkytdg lpaadlippp lkprkvwiiy sadhplyvdv 361 vlkfagflit acgtevaldl leeqaiseag vmtwvgrqkq emvesnskii vlcsrgtrak 421 wqaligrgap vrlrcdhgkp vgdlftaamr milpdfkrpa cfgtyvvcyf sevsccgdvp 481 dlfgaapryp lmdrfeevyf riqdlemfqp grmhrvgels gdnylrspgg rqlraaldrf 541 rdwqvrcpdw fecenlysad dqdapsldee vfeeplppg tgivkraplv repgsgacla 601 idplvgeegg aavaklepbl qprgqpapqp lhtlvlaaee galvaavepg pladgaavri 661 alagegeacp llgspgagrn svlflpvdpe dspigsstpm aspdllpedv rehleglmls 721 lfegslscqa qggcsrpamv ltdphtpyee eqrqsvqsdq gyisrsspqp pegltemeee 781 eeeeqdpgkp alplspedle slrslqrqll frglqknsgw dtmgsesegp sa

Figure 13

SEQ ID NO.	Accession ID	Protein name	Sequence
11	NM_002190.2	IL-17a	1 gcaggcacaa actcatccat cccagttga ttggaagaaa caacgatgac tcctgggaag 61 acctcattgg tgtcactgct actgctgctg agcctggagg ccatagtgaa ggcaggaatc 121 acaatcccac gaaatccagg atgcccaaat tctgaggaca agaacttccc ccggactgtg 181 atggtcaacc tgaacatcca taaccggaat accaatacca atcccaaaag gtcctcagat 241 tactacaacc gatccacctc acttggaat ctccaccgca atgaggaccc tgagagatat 301 ccctctgtga tctgggaggc aaagtgccgc cacttgggct gcatcaacgc tgatgggaac 361 gtggactacc acatgaactc tgtccccatc cagcaagaga tcctggtcct gcgcagggag 421 cctccacact gccccaactc ctccggctg gagaagatac tggtgtccgt gggctgcacc 481 tgtgtcaccc cgattgtcca ccatgtgcc taagagctct gggagccca cactcccaa 541 agcagttaga ctatggagag ccgacccagc ccctcaggaa ccctcatcct tcaaagacag 601 cctcatttcg gactaaactc attagagttc ttaaggcagt ttgtccaatt aaagcttcag 661 aggtaacact tggccaagat atgagatctg aattacctt cctctttcc aagaaggaag 721 gtttgactga gtaccaattt gcttcttgtt tacttttta agggctttaa gttatttatg 781 tatttaatat gccctgagat aacttgggg tataagattc catttaatg aattacctac 841 ttattttgt ttgtctttt aaagaagata agatttctggg cttgggaatt ttattattta 901 aaaggtaaaa cctgtattta tttgagctat ttaaggatct atttatgttt aagtatttag 961 aaaaaggtga aaagcacta ttatcagttc tgcctaggta aatgtaagat agaattaaat 1021 ggcagtgcaa aatttctgag tctttacaac atacggatat agtatttcct cctctttgtt 1081 tttaaaagtt ataacatggc tgaaaagaaa gattaaacct actttcatat gtattaattt 1141 aaatttgca attgttgag gtttacaag agatacagca agtctaactc tctgttccat 1201 taaaccctta taataaaatc cttctgtaat aataaagttt caaaagaaaa tgtttatttg 1261 ttctcattaa atgtatttta gcaaactcag ctcttcccta ttgggaagag ttatgcaaat 1321 tctcctataa gcaaaacaaa gcatgtcttt gagtaacaat gacctggaaa taccaaaat 1381 tccaagttct cgatttcaca tgccttcaag actgaacacc gactaaggtt ttcatactat 1441 tagccaatgc tgtagacaga agcatttga taggaataga gcaaataaga taatggccct 1501 gaggaatggc atgtcattat taaagatcat atgggaaaa tgaaacccc cccaaaatac 1561 aagaagttct gggaggagac attgtcttca gactacaatg tccagttct cccctagact 1621 caggcttcct ttggagatta aggcccctca gagatcaaca gaccaacatt tttctcttcc 1681 tcaagcaaca ctcctagggc ctggcttctg tctgatcaag gcaccacaca accagaaag 1741 gagctgatgg ggcagaacga acttaagta tgagaaaagt tcagcccaag taaaataaaa 1801 actcaatcac attcaattcc agagtagttt caagttttcac atcgtaacca ttttcgccc

Figure 13 (cont.)

12	NP_002181.1	IL-17a	1 mtpgktslvs lllllsleai vkagitiprn pgcpnsedkn fprtvmvnln ihnrntntnp 61 krssdyynrs tspwnlhrne dperypsviw eakcrhlgci nadgnvdyhm nsvpiqqeil 121 vlrrepphcp nsfrlekilv svgctcvtpi vhhva

Figure 14

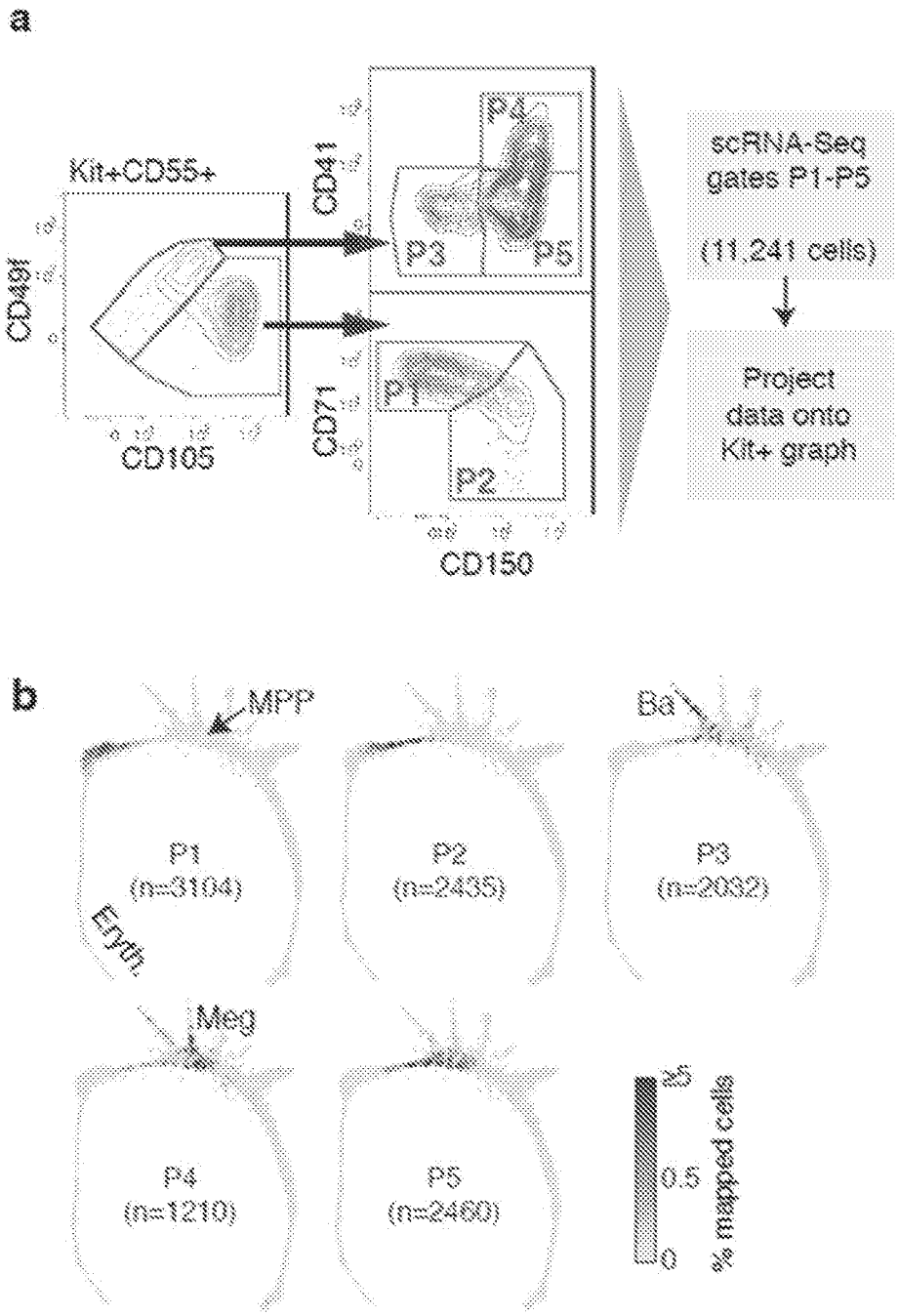
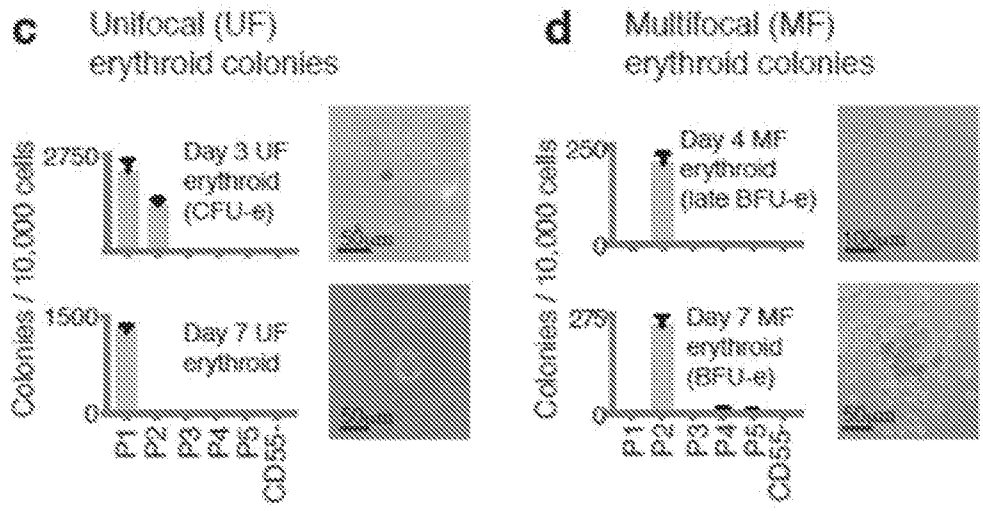
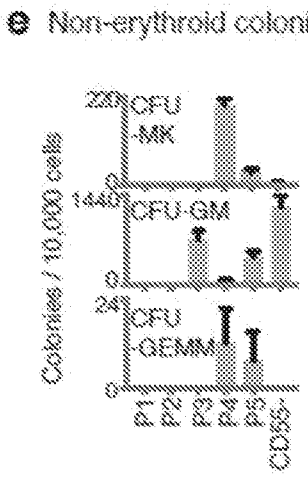

a
Kit+CD55+
CD49f
CD105
CD41
CD71
CD150
P1
P2
P3
P4
P5
scRNA-Seq gates P1-P5
(11.241 cells)
Project data onto Kit+ graph
b
MPP
Ba
Meg
Eryth
P1 (n=3104)
P2 (n=2435)
P3 (n=2032)
P4 (n=1210)
P5 (n=2460)
≥5
0.5
0
% mapped cells

Figure 14 (cont.)





f

Isolation of separate BFUs and CFUs among erythroid committed cells

	P5	P2	P1
BFU-e (MF)	-	+	-
CFU-e (UF)	-	+	++
Erythroid committed in colony assays	-	+	+

Figure 15

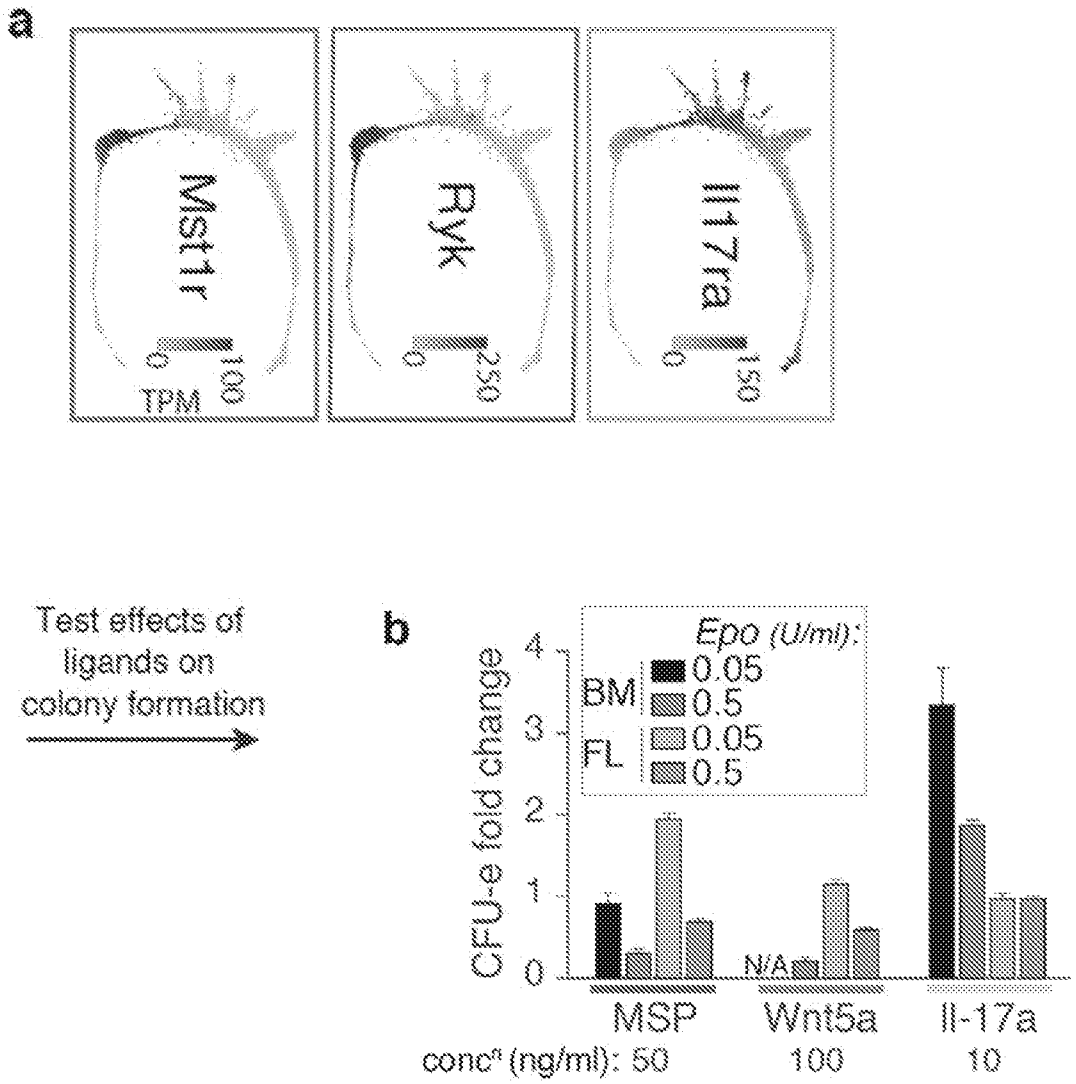
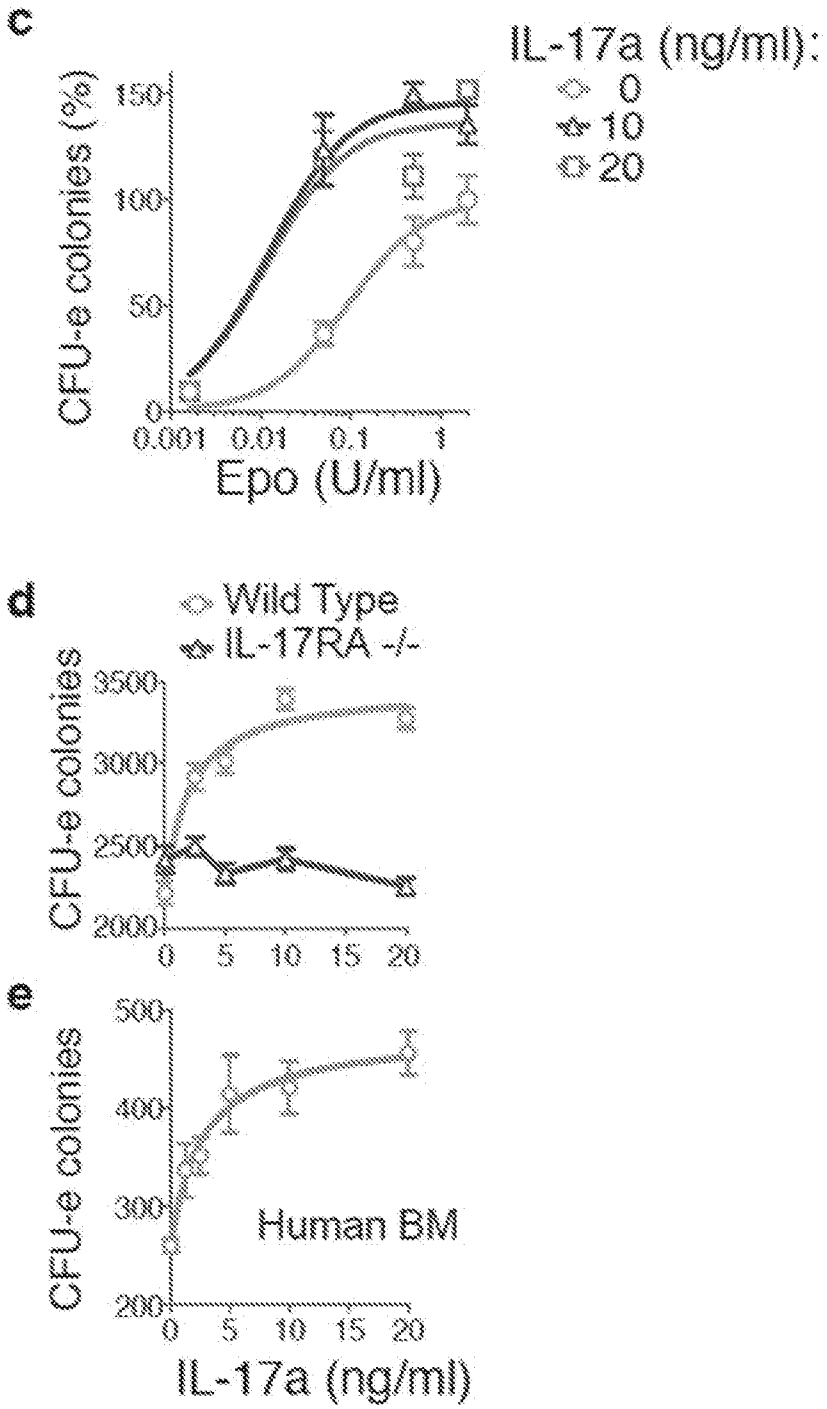

a
Mst1r
Ryk
Il17ra
0
100
250
150
TPM
Test effects of ligands on colony formation
b
Epo (U/ml):
BM
FL
0.05
0.5
0.05
0.5
CFU-e fold change
0
1
2
3
4
N/A
MSP
Wnt5a
Il-17a
conc^n (ng/ml): 50
100
10

Figure 15 (cont.)



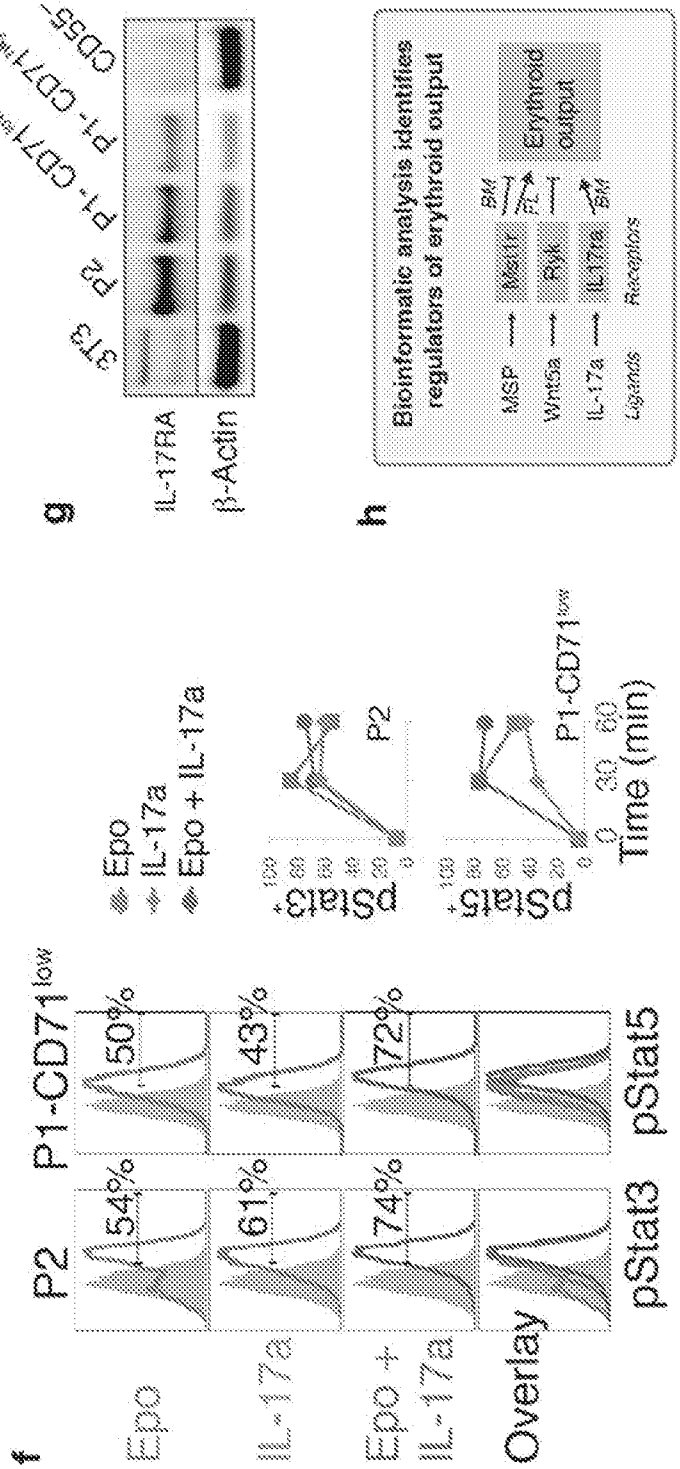
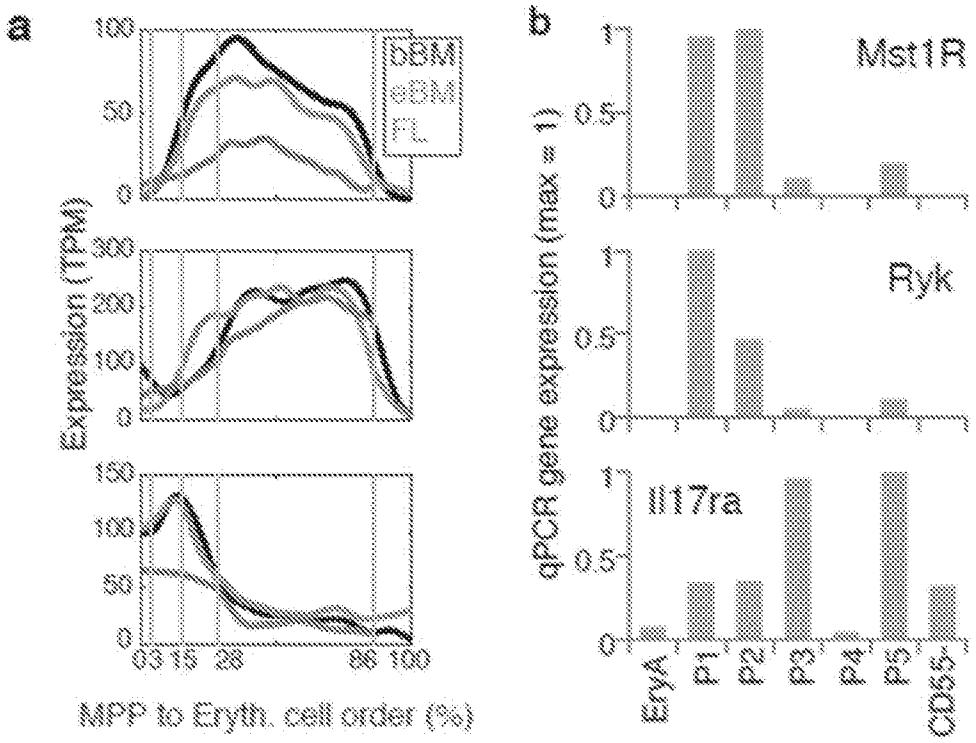


Figure 15 (cont)

Figure 16


a
100
50
0
BBM
sBM
FL
Expression (TPM)
300
200
100
0
150
100
50
0
03 15 28 86 100
MPP to Eryth. cell order (%)
b
qPCR gene expression (max = 1)
1
0.5
0
Mst1R
Ryk
Il17ra
EryA
P1
P2
P3
P4
P5
CD55-