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(54) Title: METHODS AND COMPOSITIONS FOR MAINTAINING AND EXPANDING HEMATOPOIETIC STEM CELLS

(57) Abstract: Methods for maintaining and expanding human CD34+ hematopoietic stem cells (HSCs) are provided using chemically-defined culture media that allow for expansion of HSCs in as little as six days. Culture media, isolated cell populations and kits are also provided.

METHODS AND COMPOSITIONS FOR MAINTAINING AND EXPANDING HEMATOPOIETIC STEM CELLS

Related Applications

This application claims priority to U.S. Provisional Application No. 63/284,360, filed November 30, 2021. The entire contents of which is hereby incorporated by reference.

Background of the Invention

Hematopoietic stem cells (HSCs) are pluripotent, self-renewing cells that give rise to the entire hematopoietic system, including cells of the myeloid and lymphoid lineages. HSCs are rare cells naturally found in bone marrow and umbilical cord blood, and even more rarely in peripheral blood. HSCs are typically defined by the expression, or lack of expression, of particular markers, including expression of CD34 and lack of expression of Lineage-specific markers and CD38 (Lin-CD34+CD38-). Allogeneic HSCs are used in stem cell transplantation as a means of treatment for serious hematological diseases, including cancer, such as leukemias and lymphomas, and autoimmune disorders. The ability to use HSCs in transplantation, however, requires the ability to isolate, expand and maintain HSCs in culture, given their very limited occurrence naturally.

Various culture systems for expanding CD34+ HSCs have been described, typically including a culture medium supplemented with stem cell factor (SCF) and thrombopoietin (TPO), along with other cytokines and small molecules. For example, Nishino et al. report expanding HSCs by culturing for seven days in a medium containing SCF, TPO and garcinol, a potent inhibitor of histone acetyltransferase (Nishino et al. (2011) *PLoS One* 6:e24298). Himburg et al. report expanding HSCs by culturing in media containing SCF, TPO, Flt-3 ligand and pleiotrophin, the latter of which activates PI3K signaling (Himburg et al. (2010) *Nat. Med.* 16:475-482). CD34+ HSCs from umbilical cord blood have been expanded *ex vivo* by culture in a base media containing SCF, TPO, Flt-3 ligand and low density lipoproteins to which was added an inhibitor of the JNK pathway (Xiao et al. (2019) *Cell. Discovery* 5:2). Alternatively, CD34+ HSCs from umbilical cord blood were expanded *ex vivo* by culture in a media containing SCF, TPO, Flt-3 and IL-3 for 16 hours followed by further culture for seven days with the deacetylase inhibitor valproic acid (VPA) (Papa et al. (2019) *J. Vis. Exp.* DOI:10.3791/59532).

Aryl hydrocarbon receptor antagonists, such as the purine derivative SR1, have been reported to promote the expansion of HSCs (Boitano et al. (2010) *Science* 329:1345-1348).

Antagonism of peroxisome proliferator-activated receptor (PPAR)-γ has been reported to promote the expansion of HSCs (Guo et al. (2018) *Nat. Med.* 24:360-367).

More recently, addition of an inhibitor of the NFκB signaling pathway for 10 days has been reported to increase expansion of HSCs in culture (Sun et al. (2021) *Exp. Cell. Res.* 399:112468).

Furthermore, through screening of a panel of 186 chemicals, the combination of CHIR-99021 (GSK3 inhibitor), Forskolin (cAMP activator) and OAC1 (Oct4 activator) was identified as a chemical cocktail that supported the maintenance of HSCs in culture (Jiang et al. (2018) *Cell. Discovery* 4:59).

Accordingly, while some progress has been, there remains a need for efficient and robust methods and compositions for expanding and maintaining HSCs in culture.

Summary of the Invention

This disclosure provides methods of maintaining and expanding human hematopoietic stem cells (HSCs), e.g., from cord blood or bone marrow cells, using chemically-defined culture media that allows for expansion of CD34+ HSCs in as little as six days of culture. The culture media lacks serum and comprises small molecule agents that either agonize or antagonize particular signaling pathway in stem cells such that expansion and self-renewal along the CD34+ HSC lineage is promoted, leading to expression of HSC-associated biomarkers. The methods of the disclosure have the advantage that they significantly shorten the time needed to expand CD34+ HSC. Moreover, the use of small molecule agents in the culture media allows for precise control of the culture components.

Accordingly, in one aspect, the disclosure pertains to a method of maintaining or expanding human CD34+ hematopoietic stem cells (HSCs), the method comprising culturing human CD34+ HSCs in a culture media comprising a c-kit ligand, a TPOR agonist, a TGFβ pathway agonist, an antioxidant, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist, a Notch agonist and a histone deacetylase (HDAC) inhibitor.

In an embodiment, the c-kit ligand is stem cell factor (SCF).

In an embodiment, the TPOR agonist is thrombopoietin (TPO). In other embodiments, the TPOR agonist is eltrombopag, TA-316, TPO agonist 1, avatrombopag or lusutrombopag.

In an embodiment, the TGFβ pathway agonist is Activin A. In another embodiment, the TGFβ pathway agonist is alantolactone.

In an embodiment, the antioxidant is a form of vitamin C (ascorbic acid), such as L-ascorbic acid phosphate sesquimagnesium salt hydrate, a stable form of vitamin C (Vit. C). In other embodiments, the antioxidant is ascorbic acid, glutathione, ebeselen, N-acetyl-L-cysteine or α-tocopherol.

In an embodiment, the bioactive phospholipid is lysophosphatidic acid (LPA). In other embodiments, the bioactive phospholipid is sphingosine-1-phosphage (S1P), ceramide-1-phosphate (C1P) or lysophosphatidylcholine (LPC).

In an embodiment, the AhR agonist is 6-Formylindolo[3,2-b]carbazole (FICZ). In other embodiments, the AhR agonist is Norisoboldine, Pifithrin-α hydrobromide, MeBIO, ITE or 10-Cl-BBQ.

In an embodiment, the Notch agonist is Yhhu 3792. In other embodiments, the Notch agonist is Jagged 1-2 or DLL1-4.

In an embodiment, the HDAC inhibitor is valproic acid (VPA). In other embodiments, the HDAC inhibitor is selected from the group consisting of vorinostat, entinostat, Panobinostat, Trichostatin A, mocetinostat, 4-Phenylbutyric acid, ACY-775, GSK3117391, belinostat, romidepsin, MC1568, tubastatin A, Givinostat, dacinostat, CUDC-101, quisinostat, pracinostat, PCI-34051, droxinostat, abexinostat, RGFP966, AR-42, ricolinostat, tacedinaline, fimepinostat, sodium butyrate, curcumin, M344, tubacin, RG2833, resminostat, divalproex sodium, scriptaid, sodium phenylbutyrate, tubastatin A, sinapinic acid, TMP269, CAY10683, TMP195, UF010, tasquinimod, SKLb-23bb, isoguanosine, NKL22, sulforaphane, BRD73594, citarinostat, suberohydroxamic, BRD3308, splitomicin, HPOB, LMK235, Biphenyl-4-sulfonyl chloride, nexturastat A, BML-210, TC-H106, SR-4370, TH34, Tucidinostat, SIS17, parthenolide, wt161,CAY10603, ACY738, Raddeanin A, Tinostamustine, domatinostat, BG45 and ITSA-1.

In an embodiment, the method of maintaining or expanding human CD34+ hematopoietic stem cells (HSCs) comprises culturing human CD34+ HSCs in a culture media comprising Stem Cell Factor (SCF), thrombopoietin (TPO), Activin A, Vitamin C, lysophosphatidic acid (LPA), 6-Formylindolo[3,2-b]carbazole (FICZ), Yhhu 3792 and valproic acid (VPA). In an

embodiment, SCF is present at a concentration of 10 ng/ml, TPO is present at a concentration of 100 ng/ml, Activin A is present at a concentration of 20 ng/ml, Vitamin C is present at a concentration of 100 uM, LPA is present at a concentration of 200 nM, FICZ is present at a concentration of 500 nM, Yhhu 3792 is present at a concentration of 750 nM and VPA is present at a concentration of 150 uM.

In the methods and compositions of the disclosure, the CD34+ HSCs can be from, for example, umbilical cord blood or bone marrow.

In an embodiment, the CD34+ HSCs are cultured in the media described herein for at least six days (e.g., for 7 days, or 1 week, or longer).

In an embodiment, the CD34+ HSCs have a phenotype of Lin-CD34+CD38-CD45RA-CD90+.

In another aspect, the disclosure pertains to a culture media for expanding or maintaining human CD34+ hematopoietic stem cells (HSCs) comprising a c-kit ligand, a TPOR agonist, a TGFβ pathway agonist, an antioxidant, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist, a Notch agonist and a histone deacetylase (HDAC) inhibitor.

In an embodiment, the c-kit ligand is stem cell factor (SCF).

In an embodiment, the TPOR agonist is thrombopoietin (TPO). In other embodiments, the TPOR agonist is eltrombopag, TA-316, TPO agonist 1, avatrombopag or lusutrombopag.

In an embodiment, the TGFβ pathway agonist is Activin A. In another embodiment, the TGFβ pathway agonist is alantolactone.

In an embodiment, the antioxidant is vitamin C. In other embodiments, the antioxidant is ascorbic acid, glutathione, ebeselen, N-acetyl-L-cysteine or α-tocopherol.

In an embodiment, the bioactive phospholipid is lysophosphatidic acid (LPA). In other embodiments, the bioactive phospholipid is sphingosine-1-phosphage (S1P), ceramide-1-phosphate (C1P) or lysophosphatidylcholine (LPC).

In an embodiment, the AhR agonist is 6-Formylindolo[3,2-b]carbazole (FICZ). In other embodiments, the AhR agonist is Norisoboldine, Pifithrin-α hydrobromide, MeBIO, ITE or 10-Cl-BBQ.

In an embodiment, the Notch agonist is Yhhu 3792. In other embodiments, the Notch agonist is Jagged 1-2 or DLL1-4.

In an embodiment, the HDAC inhibitor is valproic acid (VPA). In other embodiments, the HDAC inhibitor is selected from the group consisting of vorinostat, entinostat, Panobinostat, Trichostatin A, mocetinostat, 4-Phenylbutyric acid, ACY-775, GSK3117391, belinostat, romidepsin, MC1568, tubastatin A, Givinostat, dacinostat, CUDC-101, quisinostat, pracinostat, PCI-34051, droxinostat, abexinostat, RGFP966, AR-42, ricolinostat, tacedinaline, fimepinostat, sodium butyrate, curcumin, M344, tubacin, RG2833, resminostat, divalproex sodium, scriptaid, sodium phenylbutyrate, tubastatin A, sinapinic acid, TMP269, CAY10683, TMP195, UF010, tasquinimod, SKLb-23bb, isoguanosine, NKL22, sulforaphane, BRD73594, citarinostat, suberohydroxamic, BRD3308, splitomicin, HPOB, LMK235, Biphenyl-4-sulfonyl chloride, nexturastat A, BML-210, TC-H106, SR-4370, TH34, Tucidinostat, SIS17, parthenolide, wt161,CAY10603, ACY738, Raddeanin A, Tinostamustine, domatinostat, BG45 and ITSA-1.

In an embodiment, the culture media comprises Stem Cell Factor (SCF), thrombopoietin (TPO), Activin A, Vitamin C, lysophosphatidic acid (LPA), 6-Formylindolo[3,2-b]carbazole (FICZ), Yhhu 3792 and valproic acid (VPA). In an embodiment, SCF is present at a concentration of 10 ng/ml, TPO is present at a concentration of 100 ng/ml, Activin A is present at a concentration of 20 ng/ml, Vitamin C is present at a concentration of 100 uM, LPA is present at a concentration of 200 nM, FICZ is present at a concentration of 500 nM, Yhhu 3792 is present at a concentration of 750 nM and VPA is present at a concentration of 150 uM.

In yet another aspect, the disclosure pertains to an isolated cell culture of human CD34+ hematopoietic stem cells (HSCs), the culture comprising: human CD34+ HSCs cultured in a culture media comprising a c-kit ligand, a TPOR agonist, a TGFβ pathway agonist, an antioxidant, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist, a Notch agonist and a histone deacetylase (HDAC) inhibitor. Suitable agents include those described above.

In yet another aspect, the disclosure pertains to a method of maintaining human CD34+ long term hematopoietic stem cells (LT-HSCs) comprising culturing human CD34+ HSCs in a culture media comprising a TGFβ pathway agonist, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist and a histone deacetylase (HDAC) inhibitor, wherein the culture media lacks Stem Cell Factor (SCF) and thrombopoietin (TPO). Non-limiting examples of suitable agents, and concentrations therefor, include those described above. In an embodiment, the TGFβ pathway agonist is Activin A, the bioactive phospholipid is

lysophosphatidic acid (LPA), the AhR agonist is 6-Formylindolo[3,2-b]carbazole (FICZ)and the HDAC inhibitor is valproic acid (VPA). In an embodiment, the LT-HSCs are CD34+ cells that also express CRHBP, HOPX and LMO2.

In another aspect, the disclosure provides a culture media for maintaining human CD34+ long term hematopoietic stem cells (LT-HSCs) comprising a TGFβ pathway agonist, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist and a histone deacetylase (HDAC) inhibitor, wherein the culture media lacks Stem Cell Factor (SCF) and thrombopoietin (TPO). In another aspect, the disclosure provides an isolated cell culture of human CD34+ long term hematopoietic stem cells (LT-HSCs), the culture comprising: human CD34+ LT-HSCs cultured in a culture media comprising a TGFβ pathway agonist, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist and a histone deacetylase (HDAC) inhibitor, wherein the culture media lacks Stem Cell Factor (SCF) and thrombopoietin (TPO).

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

FIG. 1 shows results from an HD-DoE model of an 8-factor experiment optimized for maximum expression of GATA2. The upper section of the model shows the prediction of expression level of pre-selected 53 genes when optimized for GATA2. The lower section of the model shows the effectors that were tested in this model and their contribution to maximum expression of GATA2. The value column refers to required concentration of each effector to mimic the model.

FIG. 2 shows the dynamic profile of expression levels of GATA2, CRHBP and MEG3 genes relative to the concentration of 8 effectors tested. The positive impact of LPA, FCIZ, Vit. C and Activin A on expression of GATA2 and their factor contribution is shown by the slope of the plots for each effector.

FIG. 3 shows results from an HD-DoE model of an 8-factor experiment optimized for maximum expression of CRHBP. The upper section of the model shows the prediction of expression level of pre-selected 53 genes when optimized for CRHBP. The lower section of the model shows the effectors that were tested in this model and their contribution to maximum

expression of CRHBP. The value column refers to required concentration of each effector to mimic the model.

FIG. 4 shows the dynamic profile of expression levels of CRHBP, GATA2 and MEG3 genes relative to the concentration of 5 effectors tested. The positive impact of VPA on expression of all three genes and their factor contribution is shown by the slope of the plots for each effector.

FIG. 5 shows results from an HD-DoE model of an 8-factor experiment optimized for maximum expression of CRHBP. The upper section of the model shows the prediction of expression level of pre-selected 53 genes when optimized for CRHBP. The lower section of the model shows the effectors that were tested in this model and their contribution to maximum expression of CRHBP. The value column refers to required concentration of each effector to mimic the model.

FIGS. 6A-B show the dynamic profile of expression levels of GATA2, HOXA5 and MEG3 genes relative to the concentration of 4 validated effectors for HSC expansion (FICZ, LPA, Vit. C, Activin A). **FIG. 6A** shows expression levels of genes of interest in the presence of all five finalized effectors. **FIG. 6B** shows expression levels of genes of interest in the absence of one of the finalized effectors at a time while others are present.

FIGS. 7A-B show the dynamic profile of expression levels of GATA2, HOXA5 and MEG3 genes relative to the concentration of 1 validated effector for HSC expansion (VPA). **FIG. 7A** shows expression levels of genes of interest in the presence of VPA. **FIG. 7B** shows expression levels of genes of interest in the absence of VPA.

FIGS. 8A-B show the dynamic profile of expression levels of GATA2, HOXA5 and MEG3 genes relative to the concentration of 1 validated effector for HSC expansion (Yhhu 3792). **FIGS. 8A** shows expression levels of genes of interest in the presence of Yhhu 3792. **FIG. 8B** shows expression levels of genes of interest in the absence of Yhhu 3792.

FIGS. 9A-B show the results of flow cytometry analyses of cord blood CD34 cells grown for 7 days on the developed HSC recipe shown in Table 1 (**FIG. 9A**) or on the control recipe (SCF and TPO) (**FIG. 9B**). Cells were stained with antibodies for Lin, CD34, CD38, CD45RA, CD90 and FVS700 a live and dead marker, to exclude dead cells from the analysis,

FIG. 10 shows results from an HD-DoE model of a 12-factor experiment optimized for maximum expression of CRHBP. The upper section of the model shows the prediction of

expression level of pre-selected 53 genes when optimized for CRHBP. The lower section of the model shows the effectors that were tested in this model and their contribution to maximum expression of CRHBP. The value column refers to required concentration of each effector to mimic the model.

Detailed Description of the Invention

Described herein are methodologies and compositions that allow for maintenance and expansion of human CD34+ hematopoietic stem cells (HSCs) under chemically-defined culture conditions using a small molecule based approach. As described in Example 1, a High-Dimensional Design of Experiments (HD-DoE) approach was used to simultaneously test multiple process inputs (e.g., small molecule agonists or antagonists) on output responses, such as gene expression. These experiments allowed for the identification of chemically-defined culture media, comprising agonists and/or antagonists of particular signaling pathways, that is sufficient to maintain and expand HSCs in a very short amount of time. The optimized culture media was further validated by a factor criticality analysis, which examined the effects of eliminating individual agonist or antagonist agents, as described in Example 2. Flow cytometry analysis further confirmed the phenotype of the cells generated by the differentiation protocol, as described in Example 3. A subprotocol for maintaining long term HSCs (LT-HSCs) also was determined, as described in Example 4.

Various aspects of the invention are described in further detail in the following subsections.

I. Cells

The starting cells used in the cultures of the disclosure are human CD34+ hematopoietic stem cells. As used herein, the term "hematopoietic stem cell" (abbreviated as HSC) refers to a stem cell that has the capacity to differentiate into a variety of different hematopoietic cell types. CD34 is a transmembrane phosphoglycoprotein that has been established in the art as a surface marker for HSCs. Human HSCs are readily obtainable from available sources, including human umbilical cord blood and adult bone marrow. HSCs include both long term HSCs (LT-HSCs) and short term HSCs (ST-HSCs).

Long term HSCs (LT-HSCs) are HSCs that are found in the bone marrow or cord blood that, through a process of asymmetric cell division, can self-renew to sustain the stem cell pool or differentiate into short-term HSCs (ST-HSCs) or lineage-restricted progenitors that undergo extensive proliferation and differentiation to produce terminally differentiated cells of the blood lineage. It is believed that LT-HSCs are enriched on the fraction of Lin-CD34+CD38-CD45RA-CD90+ cells. LT-HSCs are quiescent and slow to divide in culture, taking up to 80 hours to first cell division (Cheung and Rando (2013) *Nat. Rev. Mol. Cell Biol.* 14:329-340). In contrast, short term HSCs (ST-HSCs) by definition have limited self-renewal capacity, generally described as giving rise to lymphohematopoiesis for 4–12 weeks before senescence.

In an embodiment, the HSCs express CD34 (CD34+). In an embodiment, the HSCs lack expression of the marker Lineage (Lin-). In an embodiment, the HSCs lack expression of CD38 (CD38-). In an embodiment, the HSCs lack expression of CD45RA (CD45RA-). In an embodiment, the HSCs express CD90 (CD90+). In an embodiment, the HSCs are Lin-CD34+CD38-CD45RA-CD90+ cells.

In embodiments, the HSCs express one or more genes associated with the HSC phenotype (also referred to herein as HSC-associated genetic markers), non-limiting examples of which include CHRBP, Mecom, Meg3, HOPX, LMO2, CD34, TAL1 and GATA2.

In an embodiment, the LT-HSCs are CD34+. In an embodiment, the LT-HSCs are CD34+ and also express CRHBP, HOPX and LMO2.

II. Culture Media Components

The method of the disclosure for maintaining and/or expanding CD34+ HSCs comprise culturing human CD34+ HSCs in a culture media comprising specific agonist and/or antagonists of cellular receptors and/or signaling pathways.

As described in Example 1, a culture media comprising a c-kit ligand, a TPOR agonist, a TGFβ pathway agonist, an antioxidant, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist, a Notch agonist and a histone deacetylase (HDAC) inhibitor was sufficient to maintain and expand CD34+ HSCs in as little as six days.

Additionally, as described in Example 4, a culture media comprising a TGFβ pathway agonist, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist and a histone deacetylase (HDAC) inhibitor was developed for maintaining LT-HSCs. This culture media

imparts only a low proliferative capacity to the LT-HSCs due to the lack of c-kit ligand and TPOR agonist.

As used herein, an "agonist" of a cellular receptor or signaling pathway is intended to refer to an agent that stimulates (upregulates) the cellular receptor or signaling pathway. Stimulation of the cellular signaling pathway can be initiated extracellularly, for example by use of an agonist that activates a cell surface receptor involved in the signaling pathway (e.g., the agonist can be a receptor ligand). Additionally or alternatively, stimulation of cellular signaling can be initiated intracellularly, for example by use of a small molecule agonist that interacts intracellularly with a component(s) of the signaling pathway.

As used herein, an "antagonist" of a cellular signaling pathway is intended to refer to an agent that inhibits (downregulates) the cellular signaling pathway. Inhibition of the cellular signaling pathway can be initiated extracellularly, for example by use of an antagonist that blocks a cell surface receptor involved in the signaling pathway. Additionally or alternatively, inhibition of cellular signaling can be initiated intracellularly, for example by use of a small molecule antagonist that interacts intracellularly with a component(s) of the signaling pathway.

C-kit ligands, TPOR agonists, TGFβ pathway agonists, antioxidants, bioactive phospholipids, aryl hydrocarbon receptor (AhR) agonists, Notch agonists and histone deacetylase (HDAC) inhibitors are known in the art and commercially available. They are used in the culture media at a concentration effective to achieve the desired outcome, e.g., maintenance and/or expansion of HSCs expressing markers of interest. Non-limiting examples of suitable agonist and antagonists agents, and effective concentration ranges, are described further below.

C-kit ligands include agents, molecules, compounds or substances that bind to the c-kit receptor (CD117). In an embodiment, the c-kit ligand is stem cell factor (SCF). In an embodiment, SCF is present in the media at a concentration range of 5-25 ng/ml. In an embodiment, SCF is present in the media at a concentration of 10 ng/ml.

TPOR agonists include agents, molecules, compounds or substances that agonize the thrombopoietin receptor (TPOR). In an embodiment, the TPOR agonist is thrombopoietin (TPO). In other embodiments, the TPOR agonist is eltrombopag, TA-316, TPO agonist 1, avatrombopag or lusutrombopag. In an embodiment, the TPOR agonist is TPO, which is present in the media at a concentration range of 50-150 ng/ml. In an embodiment, the TPOR agonist is TPO, which is present in the media at a concentration of 100 ng/ml.

Agonists of the TGFβ pathway include agents, molecules, compounds, or substances capable of stimulating (activating) the TGFβ signaling pathway. In an embodiment, the TGFβ pathway agonist is Activin A. In another embodiment, the TGFβ pathway agonist is alantolactone. In an embodiment, the TGFβ pathway agonist is Activin A, which is present in the media at a concentration range of 10-30 ng/ml. In an embodiment, the TGFβ pathway agonist is Activin A, which is present in the media at a concentration of 20 ng/ml.

Antioxidants include agents, molecules, compounds, or substances that prevent or slow the damage to cells caused by free radicals. In an embodiment, the antioxidant is vitamin C. In one embodiment, the vitamin C is L-ascorbic acid phosphate sesquimagnesium salt hydrate, a stable form of vitamin C (Vit. C). In other embodiments, the antioxidant is ascorbic acid, glutathione, ebeselen, N-acetyl-L-cysteine or α-tocopherol. In an embodiment, the antioxidant is vitamin C, which is present in the media at a concentration range of 50-150 uM. In an embodiment, the antioxidant is vitamin C, which is present in the media at a concentration of 100 uM.

In an embodiment, the bioactive phospholipid is lysophosphatidic acid (LPA). In other embodiments, the bioactive phospholipid is sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P) or lysophosphatidylcholine (LPC). In an embodiment, the bioactive phospholipid is LPA, which is present in the media at a concentration range of 100-300 uM. In an embodiment, the bioactive phospholipid is LPA, which is present in the media at a concentration of 200 uM.

Agonists of AhR include agents, molecules, compounds, or substances capable of stimulating (activating) the AhR signaling pathway. In an embodiment, the AhR agonist is 6-Formylindolo[3,2-b]carbazole (FICZ). In other embodiments, the AhR agonist is Norisoboldine, Pifithrin-α hydrobromide, MeBIO, ITE or 10-Cl-BBQ. In an embodiment, the AhR agonist is FICZ, which is present in the media at a concentration range of 250-750 nM. In an embodiment, the AhR agonist is FICZ, which is present in the media at a concentration of 500 nM.

Agonists of Notch include agents, molecules, compounds, or substances capable of stimulating (activating) the Notch signaling pathway. In an embodiment, the Notch agonist is Yhhu 3792. In other embodiments, the Notch agonist is Jagged 1-2 or DLL1-4. In an embodiment, the Notch agonist is Yhhu 3792, which is present in the media at a concentration

range of 500-1000 nM. In an embodiment, the Notch agonist is Yhhu 3792, which is present in the media at a concentration of 750 nM.

In an embodiment, the HDAC inhibitor is valproic acid (VPA). In other embodiments, the HDAC inhibitor is selected from the group consisting of vorinostat, entinostat, Panobinostat, Trichostatin A, mocetinostat, 4-Phenylbutyric acid, ACY-775, GSK3117391, belinostat, romidepsin, MC1568, tubastatin A, Givinostat, dacinostat, CUDC-101, quisinostat, pracinostat, PCI-34051, droxinostat, abexinostat, RGFP966, AR-42, ricolinostat, tacedinaline, fimepinostat, sodium butyrate, curcumin, M344, tubacin, RG2833, resminostat, divalproex sodium, scriptaid, sodium phenylbutyrate, tubastatin A, sinapinic acid, TMP269, CAY10683, TMP195, UF010, tasquinimod, SKLb-23bb, isoguanosine, NKL22, sulforaphane, BRD73594, citarinostat, suberohydroxamic, BRD3308, splitomicin, HPOB, LMK235, Biphenyl-4-sulfonyl chloride, nexturastat A, BML-210, TC-H106, SR-4370, TH34, Tucidinostat, SIS17, parthenolide, wt161,CAY10603, ACY738, Raddeanin A, Tinostamustine, domatinostat, BG45 and ITSA-1. In an embodiment, the HDAC inhibitor is VPA, which is present in the media at a concentration range of 100-200 uM. In an embodiment, the HDAC inhibitor is VPA, which is present in the media at a concentration of 150 uM.

In an embodiment, the method of expanding or maintaining human CD34+ hematopoietic stem cells (HSCs) comprises culturing human CD34+ HSCs in a culture media comprising Stem Cell Factor (SCF), thrombopoietin (TPO), Activin A, Vitamin C, lysophosphatidic acid (LPA), 6-Formylindolo[3,2-b]carbazole (FICZ), Yhhu 3792 and valproic acid (VPA). In an embodiment, these agents are present in the media at a concentration range as set forth above. In an embodiment, SCF is present at a concentration of 10 ng/ml, TPO is present at a concentration of 100 ng/ml, Activin A is present at a concentration of 20 ng/ml, Vitamin C is present at a concentration of 100 uM, LPA is present at a concentration of 200 nM, FICZ is present at a concentration of 500 nM, Yhhu 3792 is present at a concentration of 750 nM and VPA is present at a concentration of 150 uM.

III. Culture Conditions

In combination with the chemically-defined and optimized culture media described in subsection II above, the methods of maintaining or expanding CD34+ HSCs of the disclosure utilize standard culture conditions established in the art for cell culture. For example, cells can

be cultured at 37 °C and under 5% O_2 and 5% CO_2 conditions. A basal media can be used as the starting media to which supplemental agents can be added. For example, in an embodiment, the commercially available StemSpan™ SFEM II media is used as basal media. Cells can be cultured in standard culture vessels or plates, such as culture dishes, culture flasks or 96-well plates.

The starting CD34+ HSCs can be obtained by methodologies established in the art. Sources of human CD34+ HSCs include umbilical cord blood and bone marrow. CD34+ HSC can be obtained, for example, by standard magnetic enrichment.

To expand CD34+ HSCs, the cells are cultured in the optimized culture media for sufficient time to expand the CD34+ HSC population (i.e., increase the number of CD34+ HSCs in the culture). As described in the Examples, it has been discovered that culture of CD34+ HSCs in the optimized culture media for as little as six days was sufficient for CD34+ HSC expansion and expression of desired cellular markers.

In various embodiments, the CD34+ HSCs are cultured in the optimized culture media for sufficient time to increase the expression of at least one, and preferably a plurality of, HSC-associated genetic markers. Non-limiting examples of suitable HSC-associated genetic markers include CHRBP, Mecom, Meg3, HOPX, LMO2, CD34, TAL1 and GATA2. In embodiments, cells are cultured for sufficient time to increase the expression levels of at least two, at least three, at least four, at least five, at least six, at least seven or at least eight HSC-associated genetic markers. In an embodiment, cells are cultured for sufficient time to increase the expression level of at least one HSC-associated genetic marker (e.g., CHRBP) by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% as compared to the starting cell population. The level of expression of genetic markers in the cultured HSCs can be measured by techniques available in the art (e.g., RNAseq analysis).

In an embodiment, cells are cultured for at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days, at least 10 days, at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks or longer.

The culture media typically is changed regularly to fresh media. For example, in one embodiment, media is changed every 72 hours.

IV. Uses

The methods and compositions of the disclosure for maintaining and expanding HSCs allow for efficient and robust availability of these cell populations for a variety of uses. For example, the methods and compositions can be used in the study of HSC development and differentiation, including biology to assist in the understanding of hematopoietic-related diseases and disorders. For example, the HSCs (e.g., LT-HSCs) generated using the methods of the disclosure can be further purified according to methods established in the art using agents that bind to surface markers expressed on the cells.

Additionally, HSCs maintained and/or expanded according to the methods of the disclosure are contemplated for use in the treatment of various hematopoietic diseases and disorders, through delivery of the cells to a subject having the disease or disorder (e.g., HSC transplantation). Examples of hematopoietic diseases and disorders include, but are not limited to, cancers such as leukemias and lymphomas, blood disorders and autoimmune disorders.

V. Compositions

In other aspects, the disclosure provides compositions related to the methods of maintaining and expanding HSCs, including culture media and isolated cell cultures.

In one aspect, the disclosure provides a culture media for maintaining or expanding human CD34+ hematopoietic stem cells (HSCs) comprising a c-kit ligand, a TPOR agonist, a TGFβ pathway agonist, an antioxidant, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist, a Notch agonist and a histone deacetylase (HDAC) inhibitor. In another aspect, the disclosure provides a culture media for maintaining human CD34+ long term hematopoietic stem cells (LT-HSCs) comprising a TGFβ pathway agonist, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist and a histone deacetylase (HDAC) inhibitor. Non-limiting examples of suitable agents, and concentrations therefor, include those described in subsection II above.

In another aspect, the disclosure provides an isolated cell culture of human CD34+ hematopoietic stem cells (HSCs), the culture comprising: human CD34+ HSCs cultured in a culture media comprising a c-kit ligand, a TPOR agonist, a TGFβ pathway agonist, an antioxidant, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist, a Notch agonist and a histone deacetylase (HDAC) inhibitor. In another aspect, the disclosure provides an isolated cell culture of human CD34+ long term hematopoietic stem cells (LT-HSCs), the

culture comprising: human CD34+ LT-HSCs cultured in a culture media comprising a TGFβ pathway agonist, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist and a histone deacetylase (HDAC) inhibitor. Non-limiting examples of suitable agents, and concentrations therefor, include those described in subsection II above.

The present invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of figures and all references, patents and published patent applications cited throughout this application are expressly incorporated herein by reference.

EXAMPLES

Example 1: Culture Protocol Development for Expansion and Maintenance of Hematopoietic Stem Cells

In this example, a culture media recipe for expansion and maintenance of HSCs was developed. A recipe for expansion and maintenance of hematopoietic stem cells was developed that can be used to culture $CD34^+$ cells, for example from cord blood, and expand a population of cells referred to in the art as long-term HSC (LT-HSC) identified as $Lin^-CD34^+CD38^-CD45RA^-CD90^+$.

This example utilizes a method of High-Dimensional Design of Experiments (HD-DoE), as previously described in Bukys et al. (2020) *Iscience* 23:101346. The method employs computerized design geometries to simultaneously test multiple process inputs and offers mathematical modeling of a deep effector/response space. The method allows for finding combinatorial signaling inputs that control a complex process, such as during cell differentiation. It allows testing of multiple plausible critical process parameters, as such parameters impact output responses, such as gene expression. Because gene expression provides hallmark features of the phenotype of, for example, a human cell, the method can be applied to identify, and understand, which signaling pathways control cell fate. In the current example, the HD-DOE method was applied with the intent to find conditions for expansion and maintenance of HSCs.

Specifically, to develop the HSC recipe, the impact of agonists and antagonists of multiple signaling pathways (herein called effectors) were tested on expression of two sets of 53

pre-selected genes after a 3-day treatment. Specific genes in the measured subset are highly enriched in HSC and include *CRHBP, MLLT3, MEG3, MECOM, HOXA5* and *GATA2*. The effectors chosen include small molecules or proteins, some of which are used during *in vitro* expansion of HSC.

To test the effectors, experiments with at least 8 factors were designed that can assess the response of cells to 48 or more different combinations of effectors in a range of concentrations. To analyze the models, we focused on expression of genes that are either expressed or enriched in primitive HSC including *CRHBP, GATA2, MLLT3* and *MEG3*. The impact of each effector on gene expression level is defined by a parameter called factor contribution that is calculated for each effector during the modeling. All the experiments were conducted using a $CD34^+$ population of cells isolated from cord blood.

To identify the recipe for HSC expansion, cells were treated with various effectors for 3 days in media containing stem cell factor (SCF) and thrombopoietin (TPO) and the gene expression of cells was modeled. One model specifically, showed promising results on upregulation of *GATA2, CRHBP, HOXA5* and *MECOM* and *MEG3*. Additionally, on this model we observed *NOTCH1* upregulation and *MPO* downregulation. *MPO* is a myeloid marker highly expressed in neutrophils and other blood progenitor cells. Previous studies have shown that *NOTCH1* activation increases HSC self-renewal *in vivo* (Stier et al. (2002) *Blood* 99:2369-78).

As shown in the results summarized in **FIG. 1**, one model specifically showed promising results when optimized for maximum expression of GATA2. This model tested 8 factors: LPA, FICZ, Pyrintegrin, Laminin521, GO693, Vit.C, G-SCF, and activin A. Three of the effectors, (LPA, FICZ and Vit.C) showed positive impact on expression of genes of interest with 20, 21, and 12 factor contributions, respectively. CD44 is a receptor highly expressed in cells undergoing endothelial to hematopoietic transition, the process that is crucial for HSC generation (Oatley et al. (2020) *Nat Commun.* 11:586). Interestingly, we observed maximum levels of *CD44* when we optimized for *GATA2*.

MEG3 is highly expressed in mouse and human HSCs and is strongly down-regulated in early progenitors (Sommerkamp et el. (2019) *Sci Rep* 9:2110). In order to bring *MEG3* to its maximal expression level, we next optimized the model for maximum expression of *MEG3*. Two effectors with significant positive effect on expression of Meg3 were identified including Activin A, and Vit. C with 23 and 16 factor contributions, respectively (**FIG. 2**). Despite having a

modest factor contribution for GATA2, Activin A was one of the factors that was critical for many other HSC enriched genes including *MEG3*, *HOXA5* and *MECOM*, therefore Activin A is included in the recipe.

To further improve the recipe for HSC maintenance and expansion, additional HD-DoE experiments were performed. These data generated additional gene regulatory models that was used for preparation of the expansion protocol. The goal was to evaluate further factors to possibly increase the complexity of the signaling inputs to attain effective fate control. As previously, we focused on expression of HSC enriched genes mentioned earlier. An 8-factor experiment was performed, testing tropoelastin, lipid mixture, propionate, biotin, geltrex, N-acetyl cysteine, prostaglandin E2, and VPA. Similar to the previous experiment, $CD34^+$ cells were treated with 48 conditions of combinations of the factors for 3 days and gene expression analysis and modeling was performed.

As shown in the results summarized in **FIG. 3**, when optimized for *CRHBP*, one factor, VPA (histone deacetylase inhibitor) had significant positive impact with factor contribution of 32. Propionate, biotin and geltrex also had positive impacts but their factor contribution was less than 10 (8, 5 and 6 respectively) and N-acetyl cysteine had <1 positive factor contribution.

Optimization for *CRHBP* showed promising results on upregulation of *HOPX*, (a gene required for HSC stemness, (Lin et al. (2020) *Oncogene* 39:5112-5123), *NOTCH1*, *SPINK2* (a gene enriched in nascent HSC) and *RUNX1* (a gene required for HSC generation during embryogenesis (North et al. (2004) *Stem Cells* 22:158-68). Because propionate, biotin and geltrex showed low factor contributions in this context and it might downregulate other HSC enriched genes, we excluded these factors, maintaining VPA. When the same experiment was optimized for maximum expression of *GATA2* three effectors were identified with significant positive impact on expression of *GATA2* which include VPA, prostaglandin E2, and tropoelastin with 33, 25 and 17 factor contributions (**FIG. 4**). Because tropoelastin and prostaglandin E2 treatment downregulates other HSC enriched genes, we decided to exclude these factors but maintained VPA.

To further improve the recipe for HSC maintenance and expansion, additional HD-DoE experiments were performed. Another 8-factor experiment was performed consisting of LPA, Yhhu 3792, fibronectin, TPCA-1, GW9662, Z-vad-fmk, VEGF and activin A. Similarly to the

previous experiment, $CD34^+$ cells were treated with 48 conditions of combinations of the factors for 3 days and then gene expression analysis experiments were conducted.

As shown in the results summarized in **FIG. 5**, when optimized for *CRHBP*, one factor, Activin A (agonist of the TGF beta family) had significant positive impact with factor contribution of 26, confirming our previous results. LPA, Yhhu 3792 and fibronectin also had positive impacts with factor contribution of 8, 19 and 18 respectively. Conditions required for *CRHBP* optimization induced other HSC related genes such as *HOXA5*, *NOTCH1*, *MEG3* as well. *LMO2* and *LMO4* were also upregulated in this condition. Both genes are enriched in HSCs, however, expressed by other blood progenitors as well. In order to decrease the complexity of the recipe, we decided include only Yhhu 3792; a Notch signaling agonist.

Considering all the models analyzed, based on predicted conditions that maximize expression of HSC enriched genes such as *GATA2*, *CRHBP*, *HOXA5*, *MLLT3*, *MEG3* and *NOTCH1* we developed a complex recipe for HSC maintenance and expansion was developed that is composed of 8 effectors, as shown below in Table 1:

Table 1: HSC Maintenance and Expansion Culture Media Recipe

Effectors	Role	Concentration
Stem cell factor (SCF)	C-kit ligand	10 ng/mL
Thrombopoietin (TPO)	TPOR ligand	100 ng/mL
Activin A	TGFb pathway agonist	20 ng/mL
L-ascorbic acid phosphate sesquimagnesium salt hydrate (Vit.C)	Antioxidant	100 uM
Lysophosphatidic aid (LPA)	Bioactive phospholipid	200 nM
6-Formylindolo[3,2-b]carbazole (FICZ)	aryl hydrocarbon receptor (AhR) agonist	500 nM
Yhhu 3792	Notch agonist	750 nM
Valproic acid (VPA)	Histone deacetylase inhibitor	150 uM

Example 2: Factor Criticality Analysis of HSC Maintenance and Expansion Media

In this example, to assess the impact of elimination of each validated factor identified as described in Example 1, dynamic profile analysis was used and compared the expression level of

genes of interest in the absence of each finalized factor while others are kept present. Since the expression levels of genes of interest revealed whether the desired outcome was reachable, this factor criticality analysis revealed the extent of importance of each input effector. The results of the criticality analysis for the factors FICZ, LPA, Vit. C and Activin A are shown in **FIG. 6A-B**. The results of the criticality analysis for VPA are shown in **FIG. 7A-B**. The results of the criticality analysis for Yhhu 3792 are shown in and **FIG. 8A-B**.

For the HSC expansion recipe, each of the six finalized factors were removed while other five factors were present and the expression levels of GATA2, HOXA5, CRHBP was assessed compared to the presence of all five factors. Because SCF and TPO are cytokines impacting cell growth and survival, our recipe included these cytokines as well. When FICZ was removed, values for *GATA2* expression decreased from 642 to 512, while *HOXA5* and *MEG3* expression was unchanged. Absence of LPA resulted in reduced expression of *GATA2*, a decrease from 644 to 525. Removal of Vit.C decreased the level of *MEG3* from 82 to 53. Activin A is a critical factor for MEG3, since its removal decreases *MEG3* levels from 81 to 40. Activin A also has a positive impact on *GATA2* and *HOXA5* but at a lower extent.

VPA is a histone deacetylase inhibitor that has been used to expand HSC (Papa et al. (2018) *Blood Adv.* 2:2766–2779). In general, this class of drugs promotes chromatin opening, which can result in either up-regulation or repression of genes. VPA was included in the HSC recipe because removal of VPA decreased levels of *CRHBP* from 285 to 138. *MEG3* levels were reduced to half when VPA was removed (from 106 to 52). Finally, *GATA2* levels were also reduced upon VPA removal however to a lower extent.

The function of NOTCH pathway on hematopoiesis is complex and has some controversies (see e.g., Huang et al. (2021) *Front. Cell Biol.* DOI:10.3389/fcell.2020.606448). However, previous studies have shown that NOTCH stimulation by Delta-1 enhances the repopulation ability of human $CD34^{+}CD38^{-}$ cells. Yhhu 3792 is a new NOTCH agonist first described in 2018 (Lu et al. (2018) *Stem Cells* 36:1273-1285). The experiments herein revealed that Yhhu 3792 is a promoter of the HSC phenotype. Removal of Yhhu 3792 decreased levels of CRHBP from 202 to 169. The factor criticality analysis demonstrates that removal of any of the inputs are not critical for performance. This is expected due to the high complexity and number of the signaling inputs.

Example 3: Flow Cytometry Validation of Culture Media for Maintenance and Expansion of Cord Blood HSC

To further validate the HSC culture recipe described in Example 1, CD34+ cord blood derived cells were grown for 7 days in media comprising the ingredients shown in Table 1 and flow cytometry analysis was used to assess markers of the LT-HSC state. Markers tested included Lineage (CD3, CD14, CD16, CD19, CD20, CD56), CD34, CD38, CD45RA and CD90. Additionally, cells were grown in media with SCF and TPO as a control, commonly used by others to promote HSC expansion. Other commonly used cytokines to expand HSC such as IL3, G-CSF and IL6 were not used, since our studies showed an induction of a myeloid bias when CD34+ cells were cultured in the presence of these cytokines. 100,000 cells were plated for each condition.

After growing the cells for 7 days, flow cytometry analysis was performed. The immunophenotype of LT-HSC, namely $Lin^-CD34^+CD38^-CD45RA^-CD90^+$ was evaluated. The results are shown in **FIG. 9A-B**. Flow cytometry analysis confirmed the efficiency of the HSC recipe to promote HSC expansion and maintenance. In conditions supplying SCF + TPO (control), 18.3% of the cells were $CD45RA^-$ and $CD90^+$ (18.3% of the parent gate ($CD34^+CD38^-$)). In contrast, the complex media recipe as described in Example 1 resulted in 40.9% of cells being $CD45RA^-$ and $CD90^+$ (40.9% of the parent gate ($CD34^+CD38^-$)).

Example 4: Development of Conditions for LT-HSC Quiescent State Control

The aforementioned conditions described in Example 1 that generated improved numbers of LT-HSC were done in the context of continuous expansion. However, the feature set of the LT-HSC is invariably linked to quiescence; thus, at a critical level, the resulting expanded CD34+ cells are not identical to the resident LT-HSC population in marrow. For that reason, additional HD-DOE experiments were performed in basal media without SCF and TPO. On these experiments, the design was composed of 12 factors, including the factors on Table 1, plus SCF, TPO, FLT-3L, IGFII, Yoda-1 and cytosporin B.

One model, the results of which are summarized in **FIG. 10**, specifically showed promising results on upregulation of *CRHBP, HOPX, CD34, LMO2*. Levels of CRHBP were extremely high on this experiment, reaching the level of 1118. Interestingly, many factors

present in Table 1 were required to induce maximal CRHBP expression in the absence of cytokines, confirming the importance of these factors for cell stemness.

The best conditions to induce maximal CRHBP levels are shown below in Table 2.

Table 2

Effectors	Role	Concentration
Activin A	TGFb pathway agonist	20 ng/mL
Lysophosphatidic aid (LPA)	Bioactive phospholipid	200 nM
6-Formylindolo[3,2-b]carbazole (FICZ)	aryl hydrocarbon receptor (AhR) agonist	500 nM
Valproic acid (VPA)	Histone deacetylase inhibitor	150 uM

In order to compare the media in Table 2 with traditional methods for HSC culture and expansion (SCF+TPO), through mathematical modeling we create predictions of genes levels related to the stem cell phenotype, as well as lineage specific genes, when cultured in either traditional media (SCF+TPO) or the media of Table 2. These predicted gene levels are summarized below in Table 3:

Table 3:

		Normalized Gene Expression			
GENE	MARKER	SCF+TPO	Table 2 Recipe	SCF+TPO+Table2 recipe	Relative Expression (Table2/SCF+TPO)
CHRBP	LT-HSC	186	1113	381	5.98
Mecom	LT-HSC	30	34	59	1.1
Meg3	LT-HSC	1	187	117	187
HOPX	LT-HSC	139	325	91	2.33
LMO2	LT-HSC	494	1784	1084	3.61
CD34	LT-HSC + HSC	222	676	471	3.04
TAL1	HSC commitment	110	293	245	2.66

GATA2	HSC+myeloid (early)	619	639	412	1.03
KLF1	Erythroid	48	6	37	0.125
MPO	Myeloid lineages	3137	704	1164	0.22
MKI67	proliferation	249	53	155	0.21
BCL2L11	apoptosis	11	21	33	1.9

As expected, because the Table 2 recipe does not include SCF and TPO, proliferation (MKI67 level) was lower when cells were grown in this recipe. However, stem cell related genes such as CRHBP, MEG3, HOPX and CD34 were highly expressed in media from Table 2 as compared to SCF+TPO condition. On the other hand, KLF1 and MPO, two markers of lineage committed cells, were highly expressed in SCF+TPO conditions.

Equivalents

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

CLAIMS

1. A method of expanding or maintaining human CD34+ hematopoietic stem cells (HSCs) comprising culturing human CD34+ HSCs in a culture media comprising a c-kit ligand, a TPOR agonist, a TGFβ pathway agonist, an antioxidant, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist, a Notch agonist and a histone deacetylase (HDAC) inhibitor.

2. The method of claim 1, wherein the c-kit ligand is stem cell factor (SCF).

3. The method of claim 1, wherein the TPOR agonist is thrombopoietin (TPO).

4. The method of claim 1, wherein the TPOR agonist is eltrombopag, TA-316, TPO agonist 1, avatrombopag or lusutrombopag.

5. The method of claim 1, wherein the TGFβ pathway agonist is Activin A.

6. The method of claim 1, wherein the TGFβ pathway agonist is alantolactone.

7. The method of claim 1, wherein the antioxidant is vitamin C.

8. The method of claim 1, wherein the antioxidant is ascorbic acid, glutathione, ebeselen, N-acetyl-L-cysteine or α-tocopherol.

9. The method of claim 1, wherein the bioactive phospholipid is lysophosphatidic acid (LPA).

10. The method of claim 1, wherein the bioactive phospholipid is sphingosine-1-phosphage (S1P), ceramide-1-phosphate (C1P) or lysophosphatidylcholine (LPC).

11. The method of claim 1, wherein the AhR agonist is 6-Formylindolo[3,2-b]carbazole (FICZ).

12. The method of claim 1, wherein the AhR agonist is Norisoboldine, Pifithrin-α hydrobromide, MeBIO, ITE or 10-Cl-BBQ.

13. The method of claim 1, wherein the Notch agonist is Yhhu 3792.

14. The method of claim 1, wherein the Notch agonist is Jagged 1-2 or DLL1-4.

15. The method of claim 1, wherein the HDAC inhibitor is valproic acid (VPA).

16. The method of claim 1, wherein the HDAC inhibitor is selected from the group consisting of vorinostat, entinostat, Panobinostat, Trichostatin A, mocetinostat, 4-Phenylbutyric acid, ACY-775, GSK3117391, belinostat, romidepsin, MC1568, tubastatin A, Givinostat, dacinostat, CUDC-101, quisinostat, pracinostat, PCI-34051, droxinostat, abexinostat, RGFP966, AR-42, ricolinostat, tacedinaline, fimepinostat, sodium butyrate, curcumin, M344, tubacin, RG2833, resminostat, divalproex sodium, scriptaid, sodium phenylbutyrate, tubastatin A, sinapinic acid, TMP269, CAY10683, TMP195, UF010, tasquinimod, SKLb-23bb, isoguanosine, NKL22, sulforaphane, BRD73594, citarinostat, suberohydroxamic, BRD3308, splitomicin, HPOB, LMK235, Biphenyl-4-sulfonyl chloride, nexturastat A, BML-210, TC-H106, SR-4370, TH34, Tucidinostat, SIS17, parthenolide, wt161,CAY10603, ACY738, Raddeanin A, Tinostamustine, domatinostat, BG45 and ITSA-1.

17. The method of claims1, wherein the CD34+ HSCs are from umbilical cord blood.

18. The method of claim 1, wherein the CD34+ HSCs are from bone marrow.

19. The method of claim 1, wherein the CD34+ HSCs are cultured for at least six days.

20. The method of claim 1, wherein the CD34+ HSCs have a phenotype of Lin-CD34+CD38-CD45RA-CD90+.

21. A method of expanding or maintaining human CD34+ hematopoietic stem cells (HSCs) comprising culturing human CD34+ HSCs in a culture media comprising Stem Cell Factor (SCF), thrombopoietin (TPO), Activin A, Vitamin C, lysophosphatidic acid (LPA), 6-Formylindolo[3,2-b]carbazole (FICZ), Yhhu 3792 and valproic acid (VPA).

22. The method of claim 21, wherein SCF is present at a concentration of 10 ng/ml, TPO is present at a concentration of 100 ng/ml, Activin A is present at a concentration of 20 ng/ml, Vitamin C is present at a concentration of 100 uM, LPA is present at a concentration of 200 nM, FICZ is present at a concentration of 500 nM, Yhhu 3792 is present at a concentration of 750 nM and VPA is present at a concentration of 150 uM.

23. A culture media for expanding or maintaining human CD34+ hematopoietic stem cells (HSCs) comprising a c-kit ligand, a TPOR agonist, a TGFβ pathway agonist, an antioxidant, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist, a Notch agonist and a histone deacetylase (HDAC) inhibitor.

24. The culture media of claim 23, wherein the c-kit ligand is stem cell factor (SCF).

25. The culture media of claim 23, wherein the TPOR agonist is thrombopoietin (TPO).

26. The culture media of claim 23, wherein the TPOR agonist is eltrombopag, TA-316, TPO agonist 1, avatrombopag or lusutrombopag.

27. The culture media of claim 23, wherein the TGFβ pathway agonist is Activin A.

28. The culture media of claim 23, wherein the TGFβ pathway agonist is alantolactone.

29. The culture media of claim 23, wherein the antioxidant is vitamin C.

30. The culture media of claim 23, wherein the antioxidant is ascorbic acid, glutathione, ebeselen, N-acetyl-L-cysteine or α-tocopherol.

31. The culture media of claim 23, wherein the bioactive phospholipid is lysophosphatidic acid (LPA).

32. The culture media of claim 23, wherein the bioactive phospholipid is sphingosine-1-phosphage (S1P), ceramide-1-phosphate (C1P) or lysophosphatidylcholine (LPC).

33. The culture media of claim 23, wherein the AhR agonist is 6-Formylindolo[3,2-b]carbazole (FICZ).

34. The culture media of claim 23, wherein the AhR agonist is Norisoboldine, Pifithrin-α hydrobromide, MeBIO, ITE or 10-Cl-BBQ.

35. The culture media of claim 23, wherein the Notch agonist is Yhhu 3792.

36. The culture media of claim 23, wherein the Notch agonist is Jagged 1-2 or DLL1-4.

37. The culture media of claim 23, wherein the HDAC inhibitor is valproic acid (VPA).

38. The culture media of claim 23, wherein the HDAC inhibitor is selected from the group consisting of vorinostat, entinostat, Panobinostat, Trichostatin A, mocetinostat, 4-Phenylbutyric acid, ACY-775, GSK3117391, belinostat, romidepsin, MC1568, tubastatin A, Givinostat, dacinostat, CUDC-101, quisinostat, pracinostat, PCI-34051, droxinostat, abexinostat, RGFP966, AR-42, ricolinostat, tacedinaline, fimepinostat, sodium butyrate, curcumin, M344, tubacin, RG2833, resminostat, divalproex sodium, scriptaid, sodium phenylbutyrate, tubastatin A, sinapinic acid, TMP269, CAY10683, TMP195, UF010, tasquinimod, SKLb-23bb, isoguanosine, NKL22, sulforaphane, BRD73594, citarinostat, suberohydroxamic, BRD3308, splitomicin, HPOB, LMK235, Biphenyl-4-sulfonyl chloride, nexturastat A, BML-210, TC-H106, SR-4370, TH34, Tucidinostat, SIS17, parthenolide, wt161,CAY10603, ACY738, Raddeanin A, Tinostamustine, domatinostat, BG45 and ITSA-1.

39. The culture media of claim 23, which comprises Stem Cell Factor (SCF), thrombopoietin (TPO), Activin A, Vitamin C, lysophosphatidic acid (LPA), 6-Formylindolo[3,2-b]carbazole (FICZ), Yhhu 3792 and valproic acid (VPA).

40. The culture media of claim 39, wherein SCF is present at a concentration of 10 ng/ml, TPO is present at a concentration of 100 ng/ml, Activin A is present at a concentration of 20 ng/ml, Vitamin C is present at a concentration of 100 uM, LPA is present at a concentration of 200 nM, FICZ is present at a concentration of 500 nM, Yhhu 3792 is present at a concentration of 750 nM and VPA is present at a concentration of 150 uM.

41. An isolated cell culture of human CD34+ hematopoietic stem cells (HSCs), the culture comprising: human CD34+ HSCs cultured in a culture media comprising a c-kit ligand, a TPOR agonist, a TGFβ pathway agonist, an antioxidant, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist, a Notch agonist and a histone deacetylase (HDAC) inhibitor.

42. A method of maintaining human CD34+ long term hematopoietic stem cells (LT-HSCs) comprising culturing human CD34+ HSCs in a culture media comprising a TGFβ pathway agonist, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist and a histone deacetylase (HDAC) inhibitor, wherein the culture media lacks Stem Cell Factor (SCF) and thrombopoietin (TPO).

43. The method of claim 42, wherein the TGFβ pathway agonist is Activin A, the bioactive phospholipid is lysophosphatidic acid (LPA), the AhR agonist is 6-Formylindolo[3,2-b]carbazole (FICZ)and the HDAC inhibitor is valproic acid (VPA).

44. The method of claim 42, wherein the LT-HSCs are $CD34^+$ cells that also express CRHBP, HOPX and LMO2.

45. A culture media for maintaining human CD34+ long term hematopoietic stem cells (LT-HSCs) comprising a TGFβ pathway agonist, a bioactive phospholipid, an aryl hydrocarbon

receptor (AhR) agonist and a histone deacetylase (HDAC) inhibitor, wherein the culture media lacks Stem Cell Factor (SCF) and thrombopoietin (TPO).

46. An isolated cell culture of human CD34+ long term hematopoietic stem cells (LT-HSCs), the culture comprising: human CD34+ LT-HSCs cultured in a culture media comprising a TGFβ pathway agonist, a bioactive phospholipid, an aryl hydrocarbon receptor (AhR) agonist and a histone deacetylase (HDAC) inhibitor, wherein the culture media lacks Stem Cell Factor (SCF) and thrombopoietin (TPO).

Objective | Setpoint (#20) | Alternative setpoints

	Response	Criterion	Value	Graph	log(D)	Prob. of failure
4	CD40LG	Predicted	3.17524			
5	CD44	Predicted	795.517			
6	CDK6	Predicted	1288.68			
7	CEBPE	Predicted	7.4009			
8	CEPBA	Predicted	268.483			
9	CRHBP	Predicted	106.992			
10	CSF1R	Predicted	21.2693			
11	CSF3R	Predicted	218.082			
12	EPOR	Predicted	33.4814			
13	FES	Predicted	94.0043			
14	FOXP3	Predicted	-0.112418			
15	GATA1	Predicted	499.865			
16	GATA2	Maximize	686.491		-2.11143	1.3%
17	GATA3	Predicted	15.5737			
18	GFI1	Predicted	84.6102			
19	GYPA	Predicted	11.3508			
20	HOPX	Predicted	27.5538			
21	HOXA5	Predicted	57.5694			
22	IKZF1	Predicted	372.891			
23	IL4R	Predicted	65.9865			
24	IL7R	Predicted	1.21903			
25	IRF8	Predicted	130.416			
26	ITGA2B	Predicted	333.725			
27	KIT	Predicted	278.767			
28	KLF1	Predicted	132.643			
29	LMO2	Predicted	286.551			
30	LMO4	Predicted	411.206			
31	LTB	Predicted	6.99593			
32	MECOM	Predicted	52.8816			
33	MEG3	Predicted	74.6265			
34	MKI67	Predicted	411.63			
35	MLLT3	Predicted	109.901			
36	MME	Predicted	2.05712			
37	MPL	Predicted	47.4526			
38	MPO	Predicted	1493.29			
39	MYB	Predicted	1146.39			
40	NFE2	Predicted	468.508			
41	NOTCH1	Predicted	573.682			
42	PECAM1	Predicted	338.907			
43	PRF1	Predicted	0.888664			
44	PRG2	Predicted	756.221			
45	RUNX1	Predicted	20.89			

	Factor	Role	Value	Graph	Factor contribution
1	LPA	Free	1		20.7697
2	FICZ	Free	199.84		21.9759
3	Pyrintegrin	Free	15.6365		3.21948
4	Laminin 521	Free	1.96094		7.80477
5	GO693	Free	99.3457		1.38249
6	Vit C phosphate	Free	99.6267		12.1416
7	G-CSF	Free	0.0256309		24.8876
8	Activin A	Free	9.84967		7.81846

FIG. 1

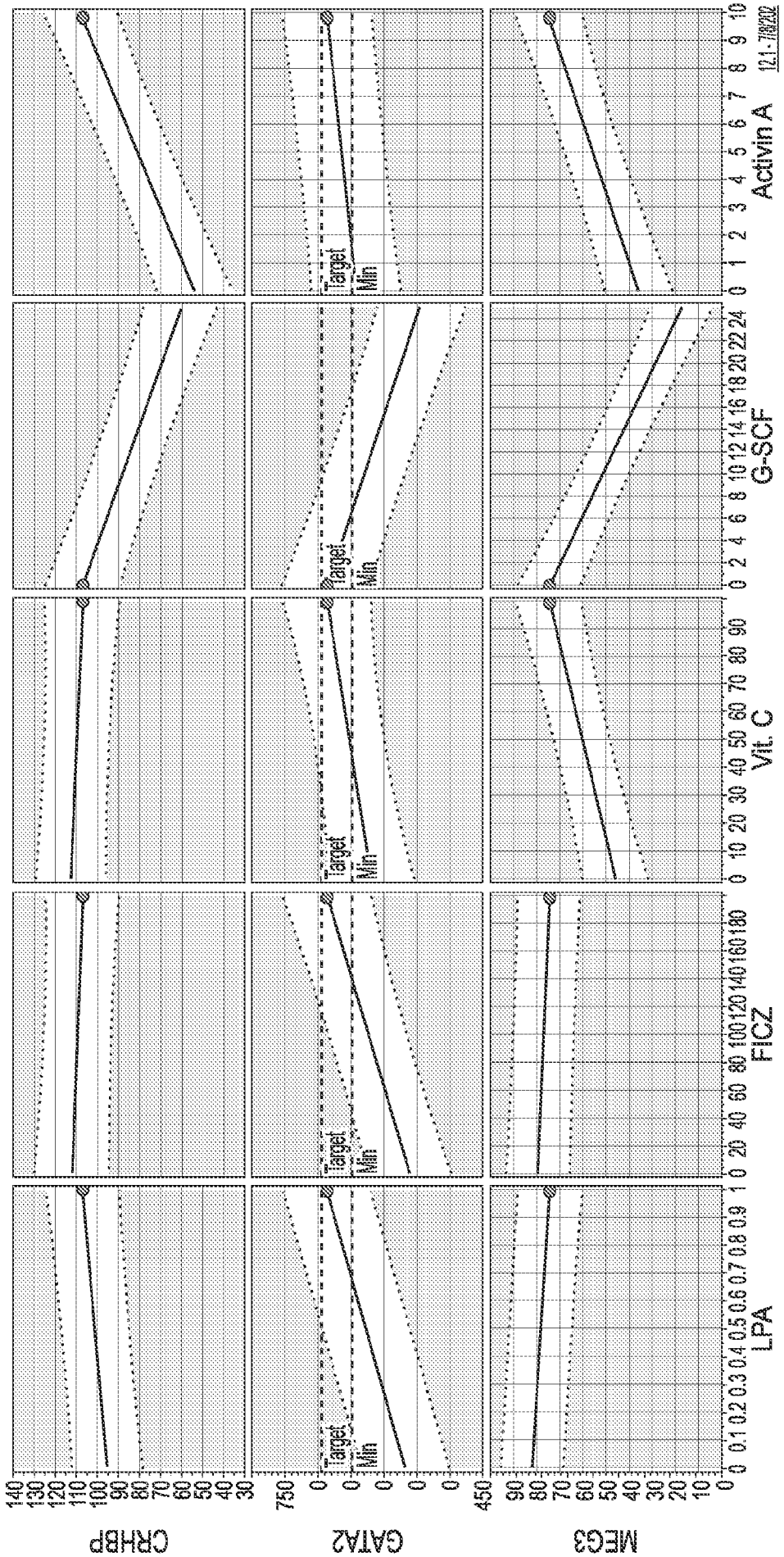


FIG. 2

Objective | Setpoint (#20) | Alternative setpoints

	Response	Criterion	Value	Graph	log(D)	Prob. of failure
8	CEBPE	Predicted	9.43529			
9	CRHBP	Maximize	369.434		-1.34147	1.2%
10	CSF1R	Predicted	3.54082			
11	CSF3R	Predicted	128.626			
12	EPOR	Predicted	40.8597			
13	FES	Predicted	217.631			
14	FOXP3	Predicted	0.0779108			
15	GATA1	Predicted	330.994			
16	GATA2	Predicted	1198.19			
17	GATA3	Predicted	33.5578			
18	GFI1	Predicted	105.631			
19	GYPA	Predicted	3.21258			
20	HOPX	Predicted	90.4613			
21	HOXA5	Predicted	34.5153			
22	IKZF1	Predicted	339.389			
23	IL4R	Predicted	139.195			
24	IL7R	Predicted	0.910902			
25	IRF8	Predicted	29.9996			
26	ITGA2B	Predicted	493.594			
27	KIT	Predicted	363.511			
28	KLF1	Predicted	160.579			
29	LMO2	Predicted	756.284			
30	LMO4	Predicted	623.298			
31	LTB	Predicted	16.0901			
32	MECOM	Predicted	18.9506			
33	MEG3	Predicted	126.963			
34	MKI67	Predicted	351.849			
35	MLLT3	Predicted	186.962			
36	MME	Predicted	1.73784			
37	MPL	Predicted	78.823			
38	MPO	Predicted	975.2			
39	MYB	Predicted	1443.75			
40	NFE2	Predicted	381.374			
41	NOTCH1	Predicted	472.744			
42	PECAM1	Predicted	331.87			
43	PRF1	Predicted	-0.354242			
44	PRG2	Predicted	223.831			
45	RUNX1	Predicted	143.318			
46	RUNX2	Predicted	100.536			
47	SPINK2	Predicted	450.83			
48	TAL1	Predicted	440.63			

	Factor	Role	Value	Graph	Factor contribution
1	TROPOELASTIN	Free	0		16.0304
2	LIPIDS	Free	0		6.94801
3	PROPIONATE	Free	0.5		8.4132
4	BIOTIN	Free	1		5.20161
5	GELTREX	Free	0.5		6.69586
6	NAcethyl cysteine	Free	100		0.895603
7	PGE2	Free	0		23.253
8	VPA	Free	1		32.5623

FIG. 3

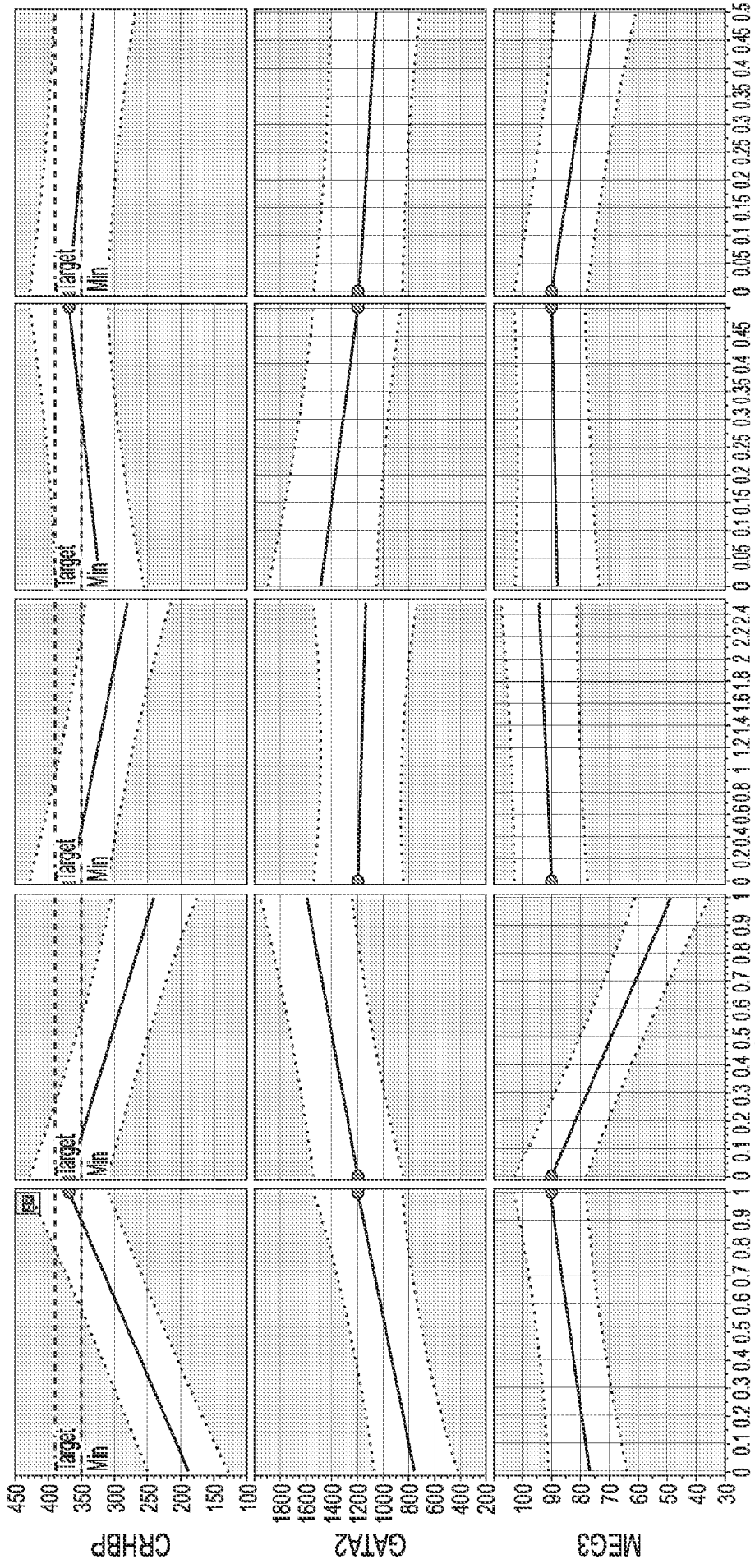

CRHBP
GATA2
MEG3
VPA
PGE2
Tropoelastin
Propionate
Lipids
Target
Min

FIG. 4

Objective | Setpoint (#20) | Alternative setpoints

	Response	Criterion	Value	Graph	log(D)	Prob. of failure
7	CEBPE	Predicted	1.66055			
8	CEBPA	Predicted	258.362			
9	CRHBP	Maximize	241.929		-10	0.49%
10	CSF1R	Predicted	4.85017			
11	CSF3R	Predicted	166.462			
12	EPOR	Predicted	16.8957			
13	FES	Predicted	161.793			
14	FOXP3	Predicted	0.717205			
15	GATA1	Predicted	368.622			
16	GATA2	Predicted	648.778			
17	GATA3	Predicted	16.2273			
18	GFI1	Predicted	95.9478			
19	GYPA	Predicted	-0.102			
20	HOPX	Predicted	78.2339			
21	HOXA5	Predicted	57.8308			
22	IKZF1	Predicted	328.989			
23	IL4R	Predicted	90.3596			
24	IL7R	Predicted	-0.239538			
25	IRF8	Predicted	66.0518			
26	ITGA2B	Predicted	379.244			
27	KIT	Predicted	253.946			
28	KLF1	Predicted	89.4802			
29	LMO2	Predicted	461.465			
30	LMO4	Predicted	307.23			
31	LTB	Predicted	10.1158			
32	MECOM	Predicted	36.7808			
33	MEG3	Predicted	49.2239			
34	MKI67	Predicted	360.908			
35	MLLT3	Predicted	193.689			
36	MME	Predicted	2.55048			
37	MPL	Predicted	78.6083			
38	MPO	Predicted	1113.21			
39	MYB	Predicted	1330.52			
40	NFE2	Predicted	346.149			
41	NOTCH1	Predicted	452.271			
42	PECAM1	Predicted	391.977			
43	PRF1	Predicted	1.46694			
44	PRG2	Predicted	232.416			
45	RUNX1	Predicted	43.0403			
46	RUNX2	Predicted	119.285			
47	SPINK2	Predicted	183.902			
48	TAL1	Predicted	167.117			

	Factor	Role	Value	Graph	Factor contribution
1	LPA	Free	0.999946		8.16246
2	Yhhu	Free	0.995907		19.5168
3	Fibronectin	Free	19.7327		18.3084
4	tpca-1	Free	11.7004		4.87282
5	GW9662	Free	3.16938		12.5138
6	Zvad	Free	92.7752		4.26584
7	VEGF	Free	0.00184975		5.98264
8	Activin A	Free	9.99809		26.377

FIG. 5

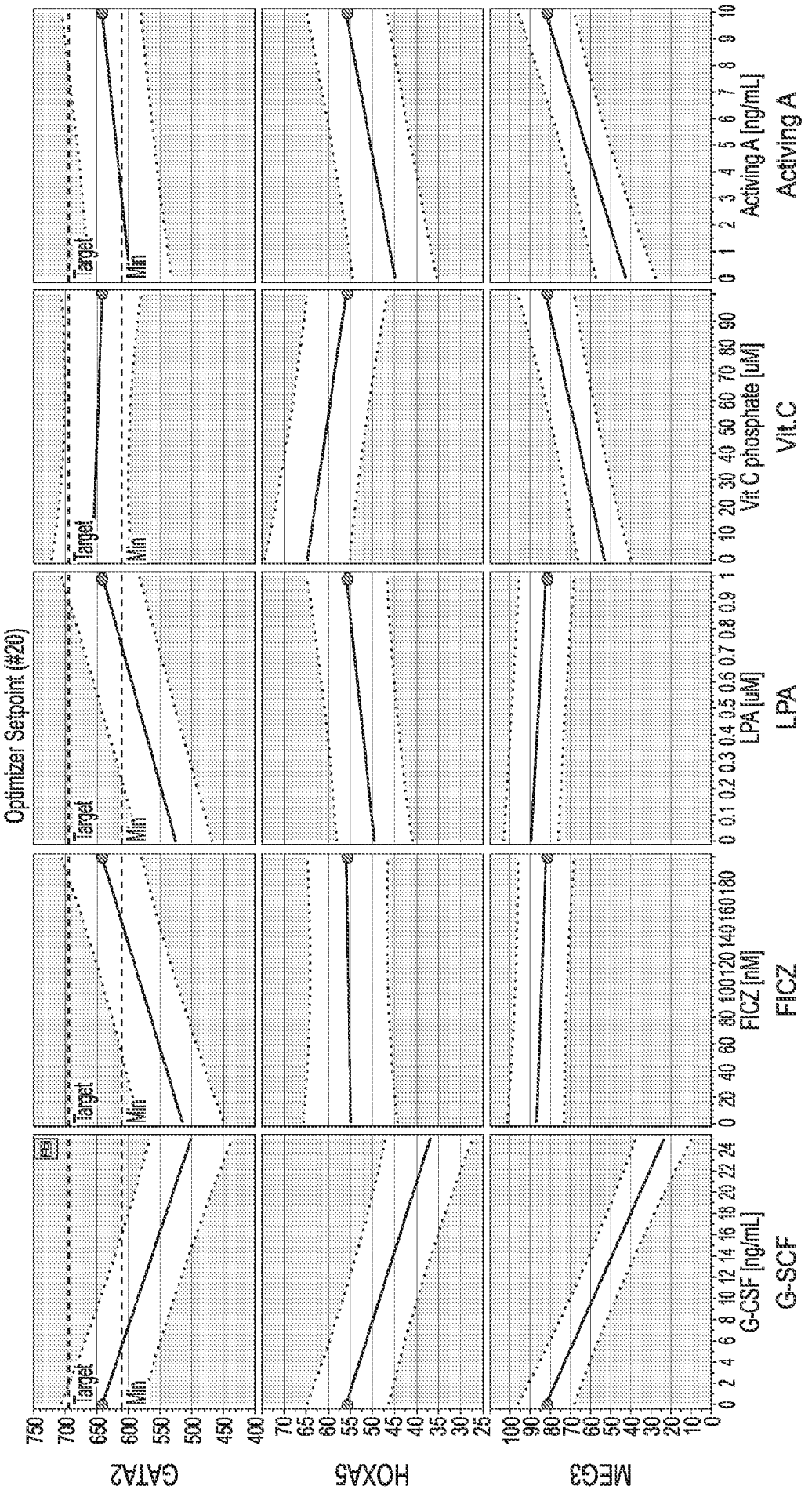

Optimizer Setpoint (#20)
GATA2
HOXA5
MEG3
Target
Min
G-CSF
FICZ
LPA
Vit.C
Activing A
G-CSF [ng/mL]
FICZ [nM]
LPA [uM]
Vit C phosphate [uM]
Activing A [ng/mL]

FIG. 6A

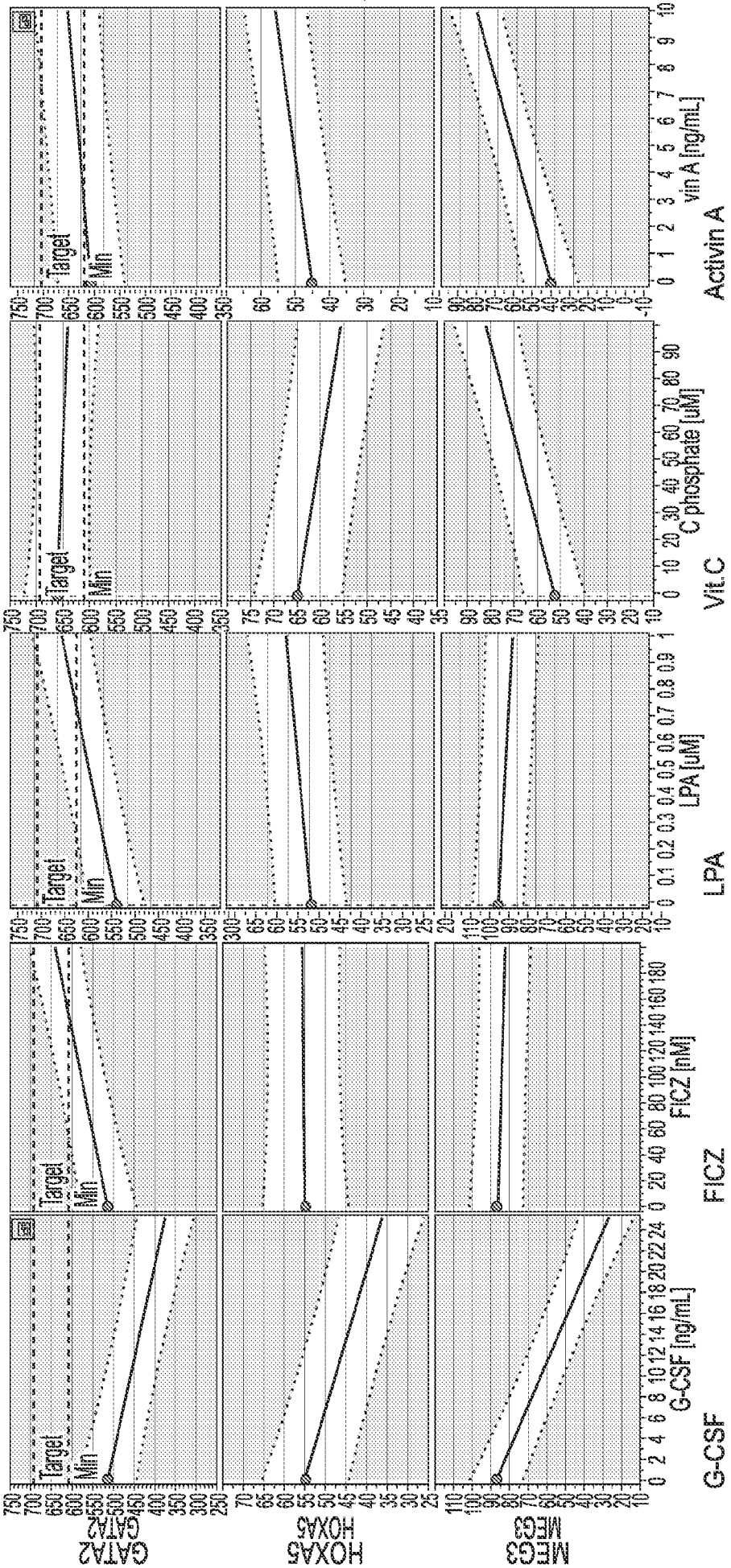

GATA2
HOXA5
MEG3
G-CSF
FICZ
LPA
Vit.C
Activin A
Target
Min
G-CSF [ng/mL]
FICZ [nM]
LPA [uM]
C phosphate [uM]
vin A [ng/mL]

FIG. 6B

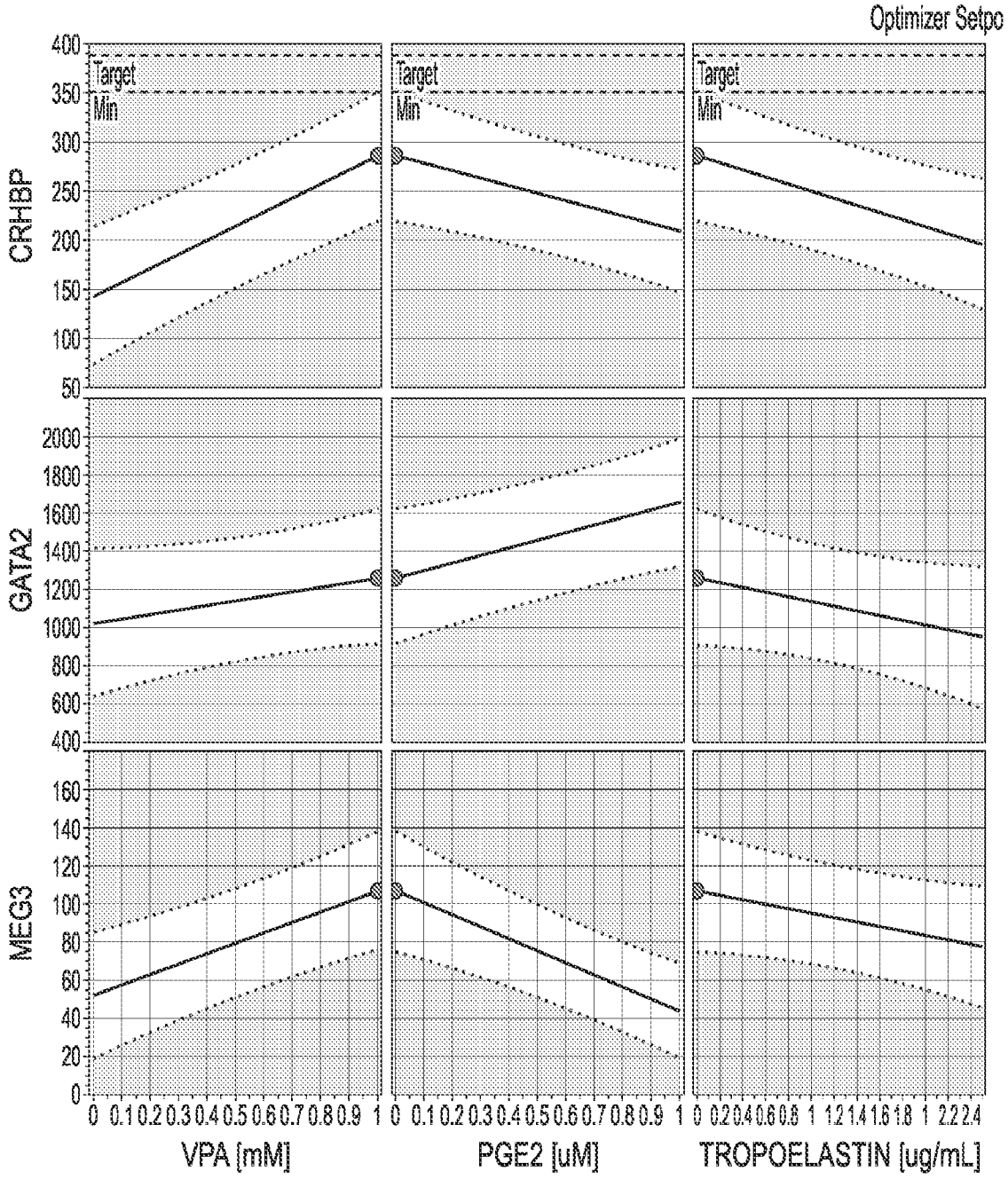

Optimizer Setpo
CRHBP
GATA2
MEG3
Target
Min
VPA [mM]
PGE2 [uM]
TROPOELASTIN [ug/mL]

FIG. 7A

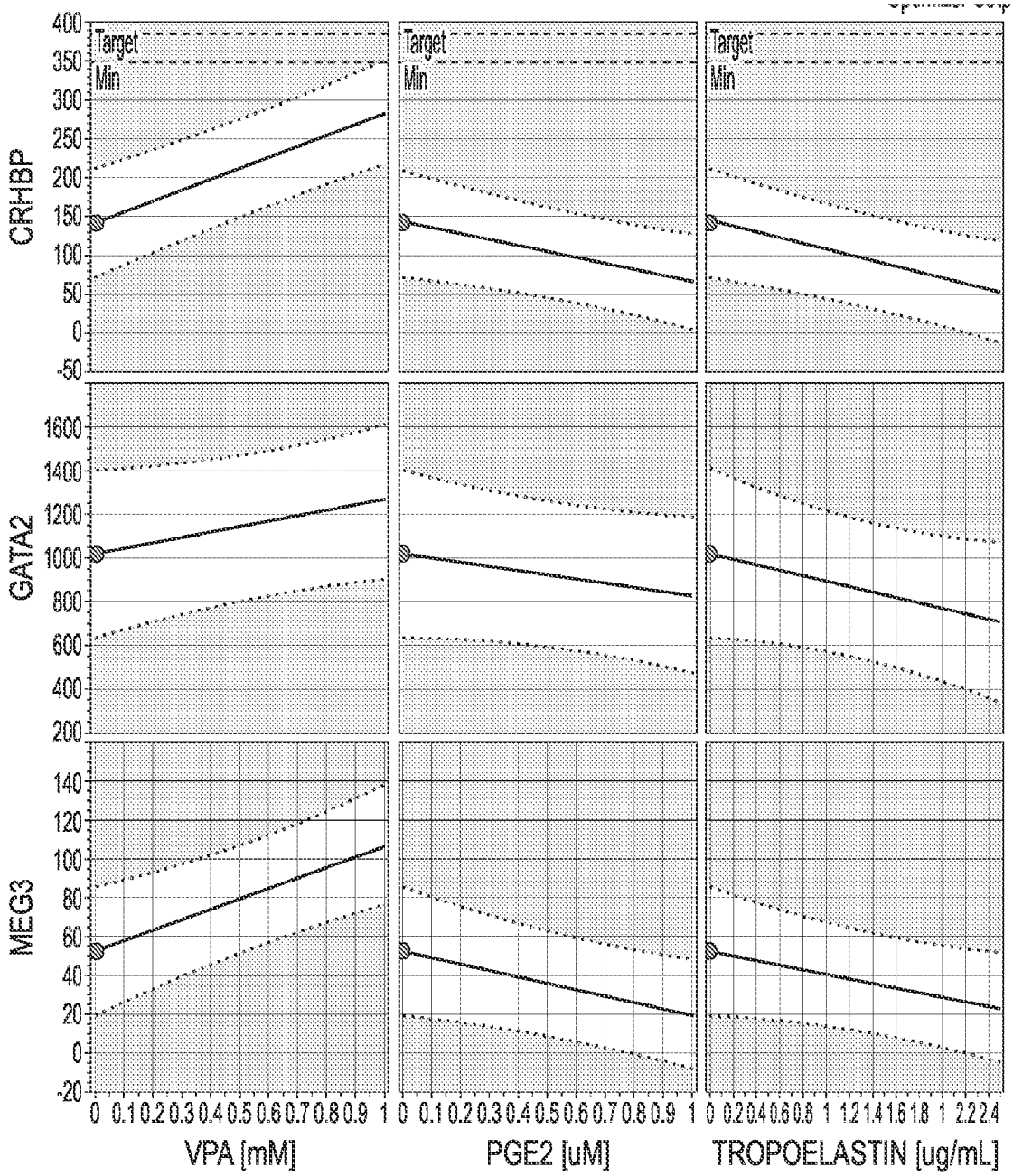

Target
Min
CRHBP
GATA2
MEG3
VPA [mM]
PGE2 [uM]
TROPOELASTIN [ug/mL]

FIG. 7B

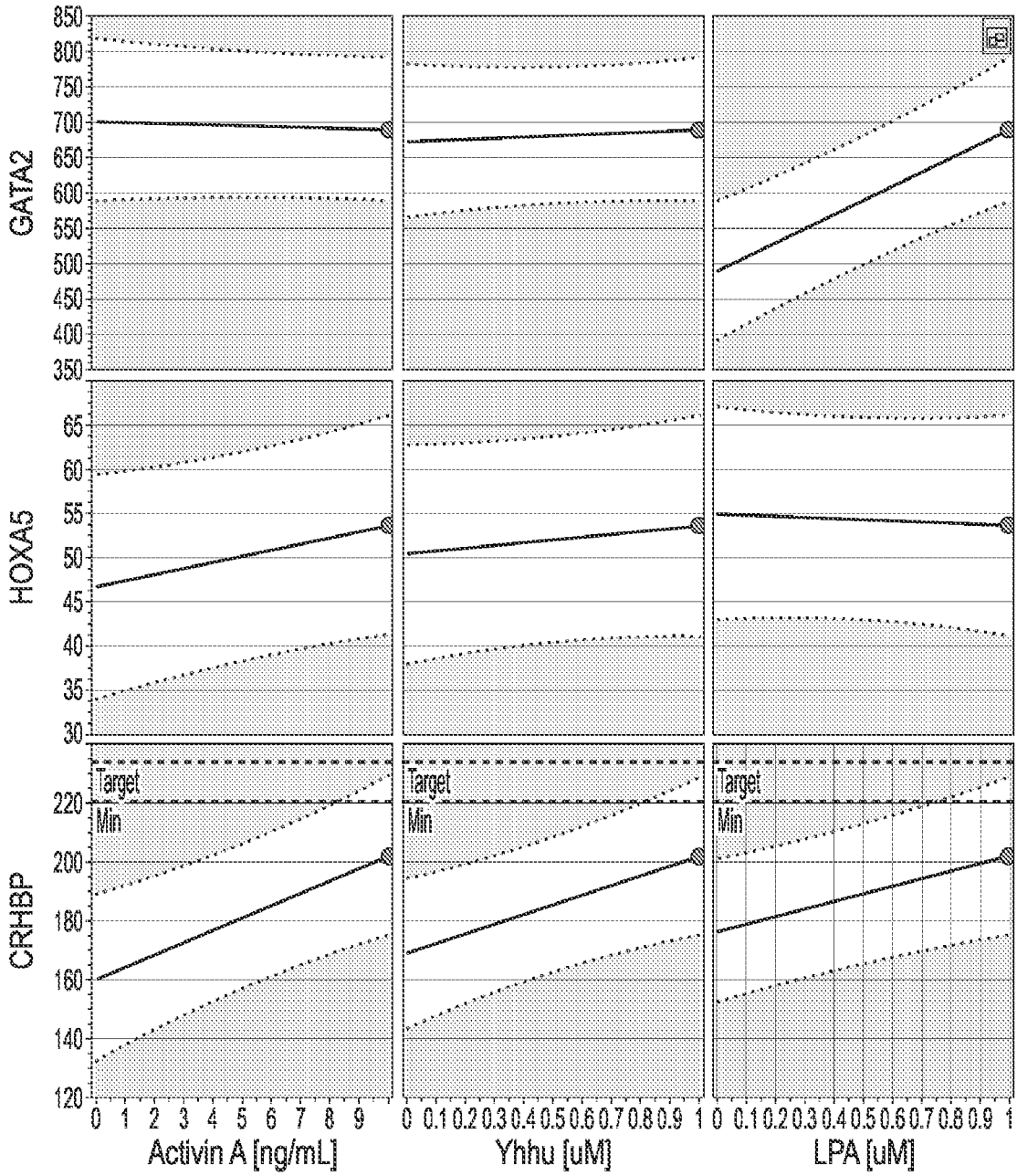


FIG. 8A

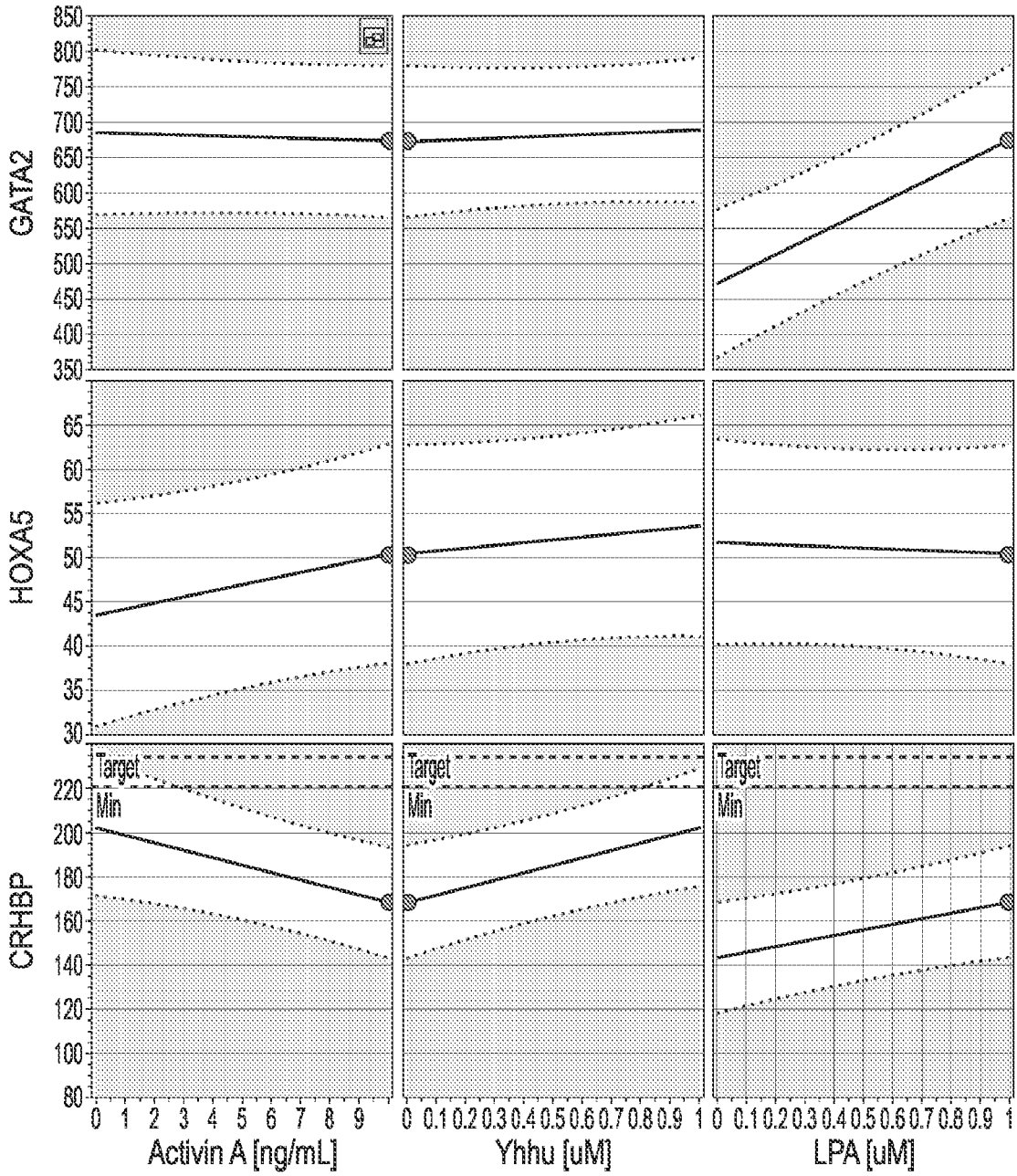


FIG. 8B

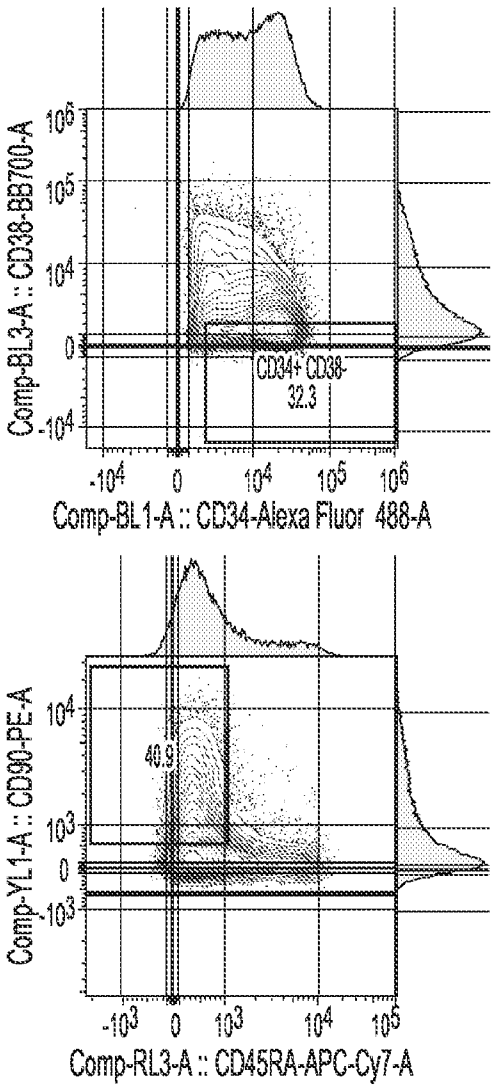

Comp-BL3-A :: CD38-BB700-A
CD34+ CD38-
32.3
Comp-BL1-A :: CD34-Alexa Fluor 488-A
Comp-YL1-A :: CD90-PE-A
40.9
Comp-RL3-A :: CD45RA-APC-Cy7-A

FIG. 9A

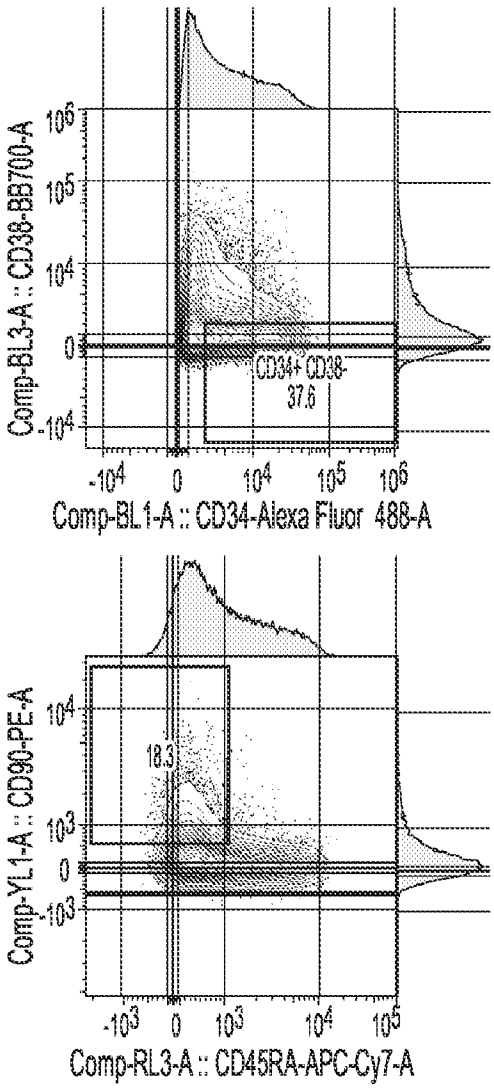

Comp-BL3-A :: CD38-BB700-A
CD34+ CD38-
37.6
Comp-BL1-A :: CD34-Alexa Fluor 488-A
Comp-YL1-A :: CD90-PE-A
18.3
Comp-RL3-A :: CD45RA-APC-Cy7-A

FIG. 9B

Objective | Setpoint (#19) | Alternative setpoints

	Response	Criterion	Value	Graph	log(D)	Prob. of failure
1	BCL2L11	Predicted	6.29755			
2	CD34	Predicted	627.665			
3	CD4	Predicted	-6.8708			
4	CD44	Predicted	534.732			
5	CDK6	Predicted	2094.57			
6	CEBPA	Predicted	542.018			
7	CEBPE	Predicted	-2.88728			
8	CRHBP	Maximize	1118.12		-1.41839	1.8%
9	CSF1R	Predicted	0.0245836			
10	CSF3R	Predicted	113.812			
11	EPOR	Predicted	26.2454			
12	FES	Predicted	98.486			
13	GATA1	Predicted	315.689			
14	GATA2	Predicted	822.827			
15	GATA3	Predicted	7.52837			
16	GFI1	Predicted	220.945			
17	HOPX	Predicted	291.972			
18	HOXA5	Predicted	23.4928			
19	IKZF1	Predicted	423.06			
20	IL4R	Predicted	70.825			
21	IRF8	Predicted	52.2296			
22	ITGA2B	Predicted	132.264			
23	KIT	Predicted	346.2			
24	KLF1	Predicted	7.71605			
25	LMO2	Predicted	1741.87			
26	LMO4	Predicted	424.876			
27	LTB	Predicted	-23.5605			
28	MECOM	Predicted	63.713			
29	MEG3	Predicted	159.579			
30	MKI67	Predicted	140.066			
31	MLLT3	Predicted	145.616			
32	MPO	Predicted	856.581			
33	MYB	Predicted	1323.52			
34	NFE2	Predicted	1147.61			
35	NOTCH1	Predicted	249.457			
36	PECAM1	Predicted	262.366			
37	PERF1	Predicted	-0.0248101			
38	PRG2	Predicted	210.126			
39	RUNX1	Predicted	62.4971			
40	RUNX2	Predicted	102.449			
41	SPINK2	Predicted	658.789			
42	TAL1	Predicted	248.492			
43	TPOR	Predicted	143.327			

	Factor	Role	Value	Graph	Factor contribution
1	FLT-3L	Free	0.00412618		13.6592
2	TPO	Free	0.0640422		7.18385
3	SCF	Free	0.000474488		21.5924
4	Activin A	Free	19.9607		5.31204
5	L-ascor Acid 2 phosphate	Free	0.103816		2.38194
6	LPA	Free	199.833		10.4309
7	FICZ	Free	499.978		9.38865
8	Yhhu	Free	0.125918		6.94138
9	VPA	Free	149.934		6.91384
10	IGFII	Free	0.000945631		9.93003
11	YODA1	Free	499.373		0.226152
12	Cytosporone B	Free	0.205715		6.03966

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/051195

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0789
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2016/210292 A1 (CHILDREN'S MEDICAL CENTER CORP [US]; RUBIN LEE [US] ET AL.) 29 December 2016 (2016-12-29) claims 1-207	1-46
Y	WO 2017/161001 A1 (CHILDREN'S MEDICAL CENTER CORP [US]) 21 September 2017 (2017-09-21) paragraphs [0143], [0256], [0315]; table 2	1-46
Y	WO 2018/197868 A1 (PLASTICELL LTD [GB]) 1 November 2018 (2018-11-01) claims 1-24	1-46

-/--

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 March 2023	15/03/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Novak-Giese, Sabine

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/051195

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DERAKHSHANI MEHDI ET AL: "Strategies for elevating hematopoietic stem cells expansion and engraftment capacity", LIFE SCIENCE, PERGAMON PRESS, OXFORD, GB, vol. 232, 25 June 2019 (2019-06-25), XP085777243, ISSN: 0024-3205, DOI: 10.1016/J.LFS.2019.116598 [retrieved on 2019-06-25] page 4, column 1, paragraph 2; figure 1 -----	1-46

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2022/051195

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		WO 2018197868 A1	01-11-2018