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May 20, 2020

Division of Dockets Management (HFA-305)
Food and Drug Administration
5630 Fishers Lane, Room 1061
Rockville, MD 20857

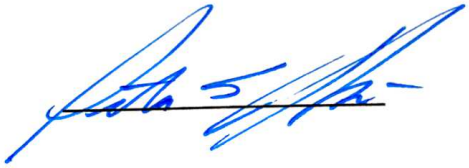
RE: Cell2in's Citizen Petition regarding the use of real-time glutathione monitoring methods as a potency assay for cell-based therapies

Dear Sir or Madam:

On behalf of Cell2in Inc., we herewith enclose a Citizen Petition and accompanying addendum of exhibits, which requests Food and Drug Administration to update its guidance documents to identify real-time glutathione monitoring methods as an acceptable means of measuring potency for purposes of submitting a Biologics License Application for cell-based therapies, including stem cell therapies, under Section 351 of the Public Health Service Act.

Please direct all correspondence relating to this petition to us at the address provided above. Thank you for your consideration of this citizen petition.

Respectfully,



Peter Choi
Emily Marden
Kelly Cho

CITIZEN PETITION RE:
USE OF REAL-TIME GLUTATHIONE MONITORING METHODS AS A POTENCY ASSAY FOR CELL-BASED THERAPIES

Submitted by Cell2in
May 20, 2020

Table of Contents

I. ACTION REQUESTED

Cell2in Inc. is a biotechnology company based in Seoul, Korea. Cell2in requests that the Food and Drug Administration (FDA) update any or all of the following guidance documents to identify real-time glutathione monitoring methods as an acceptable means of measuring potency for purposes of submitting a Biologics License Application (BLA) for cell-based therapies, including stem cell therapies, under Section 351 of the Public Health Service Act (PHS Act):

- Guidance for FDA Reviewers and Sponsors, Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs) (Apr. 2008);

- Guidance for Industry, Potency Tests for Cellular and Gene Therapy Products (Jan. 2011); and/or

- Guidance for Industry, Biologics License Applications for Minimally Manipulated, Unrelated Allogeneic Placental/Umbilical Cord Blood Intended for Hematopoietic and Immunologic Reconstitution in Patients with Disorders Affecting the Hematopoietic System (Mar. 2014).

II. STATEMENT OF GROUNDS

A. Introduction

As FDA has noted, cellular therapies are at an inflection point in medicine.[1] Research and development of cellular therapies continues to grow rapidly, and many are in clinical development. As of last year, more than 100 cellular therapies have undergone or completed clinical trials in the U.S.,[2] spanning a wide range of therapeutic products, including cellular immunotherapies, cancer vaccines, and both autologous and allogeneic cells for certain therapeutic indications, including hematopoietic stem cells and adult and embryonic stem cells.[3] Hematopoietic stem cell transplantation is currently the most common FDA-approved cellular therapy, and is used to treat a variety of disorders involved in the production of blood. FDA has also approved several chimeric antigen receptor (CAR) T-cell therapies, a type of immunotherapy that uses genetically modified T cells to treat certain types of cancers.

FDA continues to be supportive of the development of cellular therapies. At the same time the Agency has consistently made clear that quality remains a critical challenge for meeting the statutory standards of safety, purity and potency.[4] Toward this end, FDA has agreed to

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[1] FDA, FDA in Brief: FDA Advances New Efforts to Promote Development of Safe and Effective Regenerative Medicine Products (Feb. 15, 2019), https://www.fda.gov/news-events/fda-brief/fda-brief-fda-advances-new-efforts-promote-development-safe-and-effective-regenerative-medicine.

[2] PhRMA, Medicines in Development: Cell Therapy and Gene Therapy 2018 (Last visited Feb. 3, 2020) [Tab 1].

[3] FDA, Cellular & Gene Therapy Products (Jun. 20, 2019), https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products.

[4] In particular, FDA has recently expressed concern about the marketing of unsafe and ineffective stem cell therapies and has taken a number of well-publicized enforcement actions in this area in the last several years. For example, in May 2018, the Agency filed complaints seeking permanent injunctions to stop two stem cell clinics from marketing stem cell products without the Agency's approval and for their significant deviations from current good manufacturing practice (CGMP) requirements. *See* FDA, FDA

issue further manufacturing guidance for cellular therapy products[5] and has recently finalized its draft guidance on chemistry, manufacturing and control (CMC) information for Investigational New Drug Applications (INDs) for gene therapies, which includes ex vivo genetically modified cell-based gene therapies.[6] According to FDA, this category of guidances will clarify and further explain the Agency's thinking regarding critical quality attributes and other factors related to the manufacturing of cellular therapy products.

This Citizen Petition focuses on the critical quality of potency, defined in FDA regulations as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result."[7] The importance of assessing a cellular therapy product's potency cannot be overstated: without an accurate assessment of potency, there is simply no guarantee that the product will have the desired therapeutic effect in patients. The Agency has published a number of important guidance documents in the last 16 or so years containing recommendations on the design and validation of potency assays. These materials are important in providing detailed information on the application of the Agency's general rules and recommendations. But as cell-based therapies continue to grow in use and importance, further clarity would benefit the industry, especially to the extent there is uncertainty regarding the Agency's view on state-of-the-art technologies.

Accordingly, in this Citizen Petition, a method for real-time monitoring of intracellular glutathione (GSH) in living cells is identified as an assay that cell-based therapy researchers and sponsors can use to measure the potency of their products. Such an assay uses intracellular GSH as a proxy for cells' antioxidant capacity, functionality and 'stemness' – a measure of stem cells' ability to self-renew, proliferate and differentiate – without consuming the cells' GSH. Reversible and real-time GSH assays hold tremendous potential to support sponsors in their development of meaningful and relevant potency assays for the production

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Seeks Permanent Injunctions Against Two Stem Cell Clinics (May 9, 2018), https://www.fda.gov/news-events/press-announcements/fda-seeks-permanent-injunctions-against-two-stem-cell-clinics. Further, in December 2018 and April 2019, FDA sent letters to over 40 stem clinics offering stem cell products for potentially a variety of unapproved uses, reiterating that their products are regulated by the Agency and that they should comply with all agency regulations regarding Investigational New Drug (IND) application and premarket approval by November 2020, when the enforcement discretion would end. *See* FDA, Statement by FDA Commissioner Scott Gottlieb, M.D., and Biologics Center Director Peter Marks, M.D., Ph.D. on FDA's Continued Efforts to Stop Stem Cell Clinics and Manufacturers from Marketing Unapproved Products That Put Patients at Risk, While Progressing the Agency's Commitment to Help Advance Legitimate Stem Cell Product Development under Existing Agency Regulations (Apr. 3, 2019), https://www.fda.gov/news-events/press-announcements/statement-fda-commissioner-scott-gottlieb-md-and-biologics-center-director-peter-marks-md-phd-fdas; FDA, FDA Sends Warning to Company for Marketing Dangerous Unapproved Stem Cell Products that Put Patients at Risk and Puts Other Stem Cell Firms, Providers on Notice (Dec. 20, 2018), https://www.fda.gov/news-events/press-announcements/fda-sends-warning-company-marketing-dangerous-unapproved-stem-cell-products-put-patients-risk-and; Denise Grady, 12 People Hospitalized with Infections from Stem Cell Shots, NY TIMES (Dec. 20, 2018) [Tab 2].

[5] FDA, Statement from FDA Commissioner Scott Gottlieb, M.D. and Peter Marks, M.D., Ph.D., Director of the Center for Biologics Evaluation and Research on New Policies to Advance Development of Safe and Effective Cell and Gene Therapies (Jan. 15, 2019), https://www.fda.gov/news-events/press-announcements/statement-fda-commissioner-scott-gottlieb-md-and-peter-marks-md-phd-director-center-biologics.

[6] FDA, FDA Continues Strong Support of Innovation in Development of Gene Therapy Products (Jan. 28, 2020), https://www.fda.gov/news-events/press-announcements/fda-continues-strong-support-innovation-development-gene-therapy-products.

[7] 21 C.F.R. § 600.3(s).

and control of their cellular therapies through various phases of product development. The assays can also be developed to meet the applicable regulations and various criteria FDA has outlined for potency assays. As such, GSH monitoring assays meet the twin goals of ensuring patient safety and supporting innovation.

Cell2in Inc., a pioneering biotechnology company focused on the development of cell quality monitoring techniques, has been actively developing its own version of a reversible and real-time GSH monitoring assay (*i.e.*, "fluorescent real-time thiol tracer" or "FreSHtracer"). In this Citizen Petition, Cell2in presents various in vitro and in vivo data obtained using FreSHtracer to demonstrate that GSH assays can be used to measure cells' antioxidant capacity, functionality, stemness, and consequently, potency.

As discussed below, there is precedent for Agency guidance documents to identify specific assays for potency measurements. For example, the Agency's Guidance for Industry, Biologics License Applications for Minimally Manipulated, Unrelated Allogeneic Placental/Umbilical Cord Blood Intended for Hematopoietic and Immunologic Reconstitution in Patients with Disorders Affecting the Hematopoietic System (hereinafter, the "HPC, Cord Blood Guidance") identifies three specific assays: total nucleated cell count, viable nucleated cells and viable CD34+ cells.[8] Consistent with views voiced by others, the Agency's Cellular, Tissue and Gene Therapies Advisory Committee, however, has recognized that these assays have limitations and that new methods are necessary.

For the specific reasons that follow, Cell2in respectfully requests that the Agency consider the scientific information submitted in this Citizen Petition and update its guidance documents to identify real-time GSH monitoring methods, such as FreSHtracer, as an acceptable means of measuring potency for purposes of submitting a BLA for cell-based therapies, including stem cell therapies, under Section 351 of the PHS Act.

B. Technical Background Regarding Reversible and Real-Time GSH Assays

Stem cells are defined by their ability to self-renew and generate differentiated progeny. Although stem cells in different microenvironments and niches have distinct molecular programs, there are genetic traits and molecular processes – both specific to and shared by all stem cells – that give rise to these core stem cell properties.[9] The concept of 'stemness' refers to these core intrinsic properties as well as the shared genetic and molecular traits and processes.[10] For example, more primitive stem cells that are capable of self-renewing and differentiating into different types of cells are said to possess greater stemness relative to their differentiated progeny.[11]

Reactive oxygen species-mediated oxidation impacts core stem cell properties, including the cells' ability to self-renew, proliferate and differentiate. Reactive oxygen species (ROS) are

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[8] FDA, Guidance for Industry, Biologics License Applications for Minimally Manipulated, Unrelated Allogeneic Placental/Umbilical Cord Blood Intended for Hematopoietic and Immunologic Reconstitution in Patients with Disorders Affecting the Hematopoietic System, at 11 and 38 (Mar. 2014), https://www.fda.gov/media/86387/download.

[9] *See, e.g.*, D. Melton, 'Stemness': Definitions, Criteria, and Standards, Chapter 2 (R. Lanza & A. Atala eds.: Essentials of Stem Cell Biology, 3rd ed. 2014) [Tab 3].

[10] *Id.* [Tab 3]; P. Aponte and A. Caicedo, Stemness in Cancer: Stem Cells, Cancer Stem Cells, and Their Microenvironment, STEM CELLS INT. (2017) [Tab 4]; M. Pavlović and K. Radotić, Stemness and Stem Cell Markers (Animal and Plant Stem Cells, Concepts, Propagation and Engineering, Springer, Cham 2017) [Tab 5].

[11] *See*, *e.g.*, HemoGenix, The Science and Biology of Hematopoietic Stem Cell Potency, Quality and Release Criteria for Transplantation, at 2 (Oct. 2010) [Tab 6].

radical or non-radical oxygen species created by the partial reduction of oxygen. Examples of ROS include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (•OH). ROS are highly unstable and reactive molecules that oxidize biological molecules to cause damage in the human body. The body maintains cellular redox homeostasis by using antioxidants to eliminate ROS. When the redox balance is disrupted, oxidative stress increases and can lead to aging as well as various age-related degenerative diseases, including degenerative arthritis, cataract, Alzheimer's disease, cancers, fibrosis diseases, and metabolic syndromes.[12]

In stem cells (SCs), ROS are involved in regulating the balance between self-renewal and differentiation. An increase in ROS commits SCs to differentiate into various cell types[13] and exhibits inhibitory effects on SC functions.[14] These effects are mediated by oxidation-induced regulation of a variety of signaling proteins implicated in the core stem cell properties, such as OCT4, NRF2, FoxOs, APE1/Ref-1, ATM, HIF-1, p38 and p53.[15] As an example, OCT4, a pluripotency-related transcription factor, is inactivated by cysteine oxidation under glutamine-depleted conditions, which favors the differentiation and functional maturation of embryonic stem cells.[16] Disruption of NRF2, a master regulator of redox homeostasis, impairs self-renewal and pluripotency capabilities of embryonic and adult stem cells; the migration and retention of hematopoietic stem cells in the bone marrow niche; and the proliferation and homeostasis of intestinal and airway basal stem cells. Low ROS levels are critical for SCs to preserve their self-renewal process[17] as well as to prevent mutations and other damage, which can impair genetic and epigenetic integrity.[18]

Glutathione (GSH) is a naturally-produced antioxidant that plays a pivotal role in cellular redox homeostasis and defense against cellular damage from ROS. GSH is a tripeptide consisting of glutamate, cysteine and glycine with a thiol group attached to the cysteine. It is the most abundant non-protein thiol in cells, existing in millimolar concentrations. GSH is synthesized in the cytosol and transported to subcellular organelles such as mitochondria, nucleus and endoplasmic reticulum. These organelles are sensitive to oxidation and require redox buffering by GSH for their biological function. GSH accomplishes this by regulating the intensity and duration of ROS-induced signals and directly eliminating various peroxides such as hydrogen peroxide. Specifically, GSH eliminates hydrogen peroxide through a glutathione peroxidase-catalyzed reaction, producing water and oxidized GSH (GSSG). GSSG is then regenerated to GSH by glutathione reductase and NADPH.

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[12] *See*, *e.g.*, B. Uttara et al., Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options, 7 CURRENT NEUROPHARMACOLOGY, 65-74 (2009) [Tab 7]; D.M. Townsend, et al., The Importance of Glutathione in Human Disease, 57 BIOMED PHARMACOTHER., 145-155 (2003) [Tab 8].

[13] *See* C.L. Bigarella et al., Stem Cells and the Impact of ROS Signaling, 141 DEVELOPMENT, 4206-4218 (2014) [Tab 9].

[14] *See* J. Kim, and P.K.Y. Wong, Loss of ATM Impairs Proliferation of Neural Stem Cells Through Oxidative Stress-Mediated P38 MAPK Signaling, 27 STEM CELLS, 1987-1998 (2009) [Tab 10]; B. Nakamura et al., Knockout of the Transcription Factor NRF2 Disrupts Spermatogenesis in an Age-Dependent Manner, 49 FREE RADIC. BIOL. AND MED., 1368-1379 (2010) [Tab 11].

[15] C.L. Bigarella et al., *supra* note 13 [Tab 9]; K. Wang, et al., Redox Homeostasis: The Linchpin in Stem Cell Self-renewal and Differentiation, 4 CELL DEATH DIS., e537 (2013) [Tab 12].

[16] G. Marsboom, et al., Glutamine Metabolism Regulates the Pluripotency Transcription Factor OCT4, 16 CELL REP, 323–332 (2016) [Tab 13].

[17] K. Ito, and T. Suda, Metabolic Requirements for the Maintenance of Self-Renewing Stem Cells, 15 NAT. REV. MOL. CELL BIOL., 243-256 (2014) [Tab 14].

[18] *See* C.L. Bigarella et al., *supra* note 13 [Tab 9].

Due to the central role of GSH in cellular redox homeostasis, cellular GSH levels have long been used as a biomarker for estimating the degree of oxidative stress in the human body.[19] However, the lack of appropriate tools has until now made it difficult to study how GSH affects the redox balance in cells, and the exact mechanism behind the GSH-based redox system remains yet to be fully elucidated. As further explained below, recent technology advances have given rise to GSH probes and detection methods, such as Cell2in's FreSHtracer, that can address this challenge by measuring GSH concentrations and dynamics in intact living cells in real time.

1. FreSHtracer Demonstrates That GSH Levels Can Be Measured and Monitored In A Ratiometric Manner In Intact, Living Cells

Cell2in's technology, "FreSHtracer", consists of a fluorescent dye in the form of a coumarin derivative bearing a conjugated 2-cyanoacrylamide group (*see* Fig. 1 below). FreSHtracer is able to penetrate through the membranes of cells and cellular components and bind to intracellular thiols, including thiol groups on GSH.[20] When the binding occurs, the fluorescence spectrum of FreSHtracer shifts, with increased fluorescence intensity at 510 nm (F_{510}) and decreased emission intensity at 580 nm (F_{580}). Unlike other chemical probes, which undergo irreversible reactions with GSH, FreSHtracer reacts reversibly with thiols in the intracellular environment. Binding can be reversed by thiol-specific oxidants such as diamide, N-ethylmaleimide (NEM) and H_2O_2, leading to baseline fluorescence readings (*see* Fig. 2 below). Confocal microscopy and flow cytometry can be used to quantify GSH concentrations.

Cell2in has demonstrated that the ratio of F_{510} / F_{580} (FR) directly correlates with GSH concentration with a R-squared value of 0.9938. The correlation was substantiated in a concentration range wider than the concentration of GSH usually present in cells (0 to 50 mM). Although FreSHtracer also binds to other protein thiols (PSH) in cells, the FR is mainly dependent on GSH concentration and not significantly affected by typical protein concentrations in intact cells, which range from 50 to 200 mg/mL.[21] Moreover, FreSHtracer reacts rapidly with GSH, reaching equilibrium within 5 minutes, but reacts 8- to 12- fold slower with PSH. PSH-induced FreSHtracer also is unaffected by treatment with H_2O_2, suggesting that FreSHtracer can be used to selectively monitor for ROS-induced GSH changes. These results collectively demonstrate that intracellular GSH levels can be measured in a ratiometric manner, using probes such as FreSHtracer.

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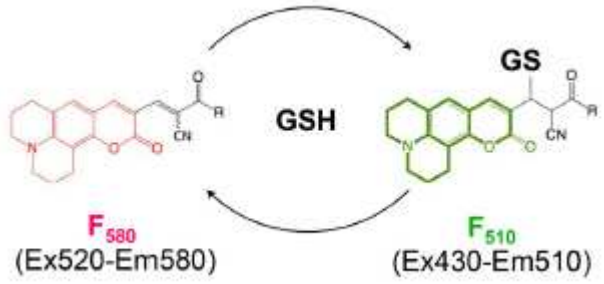
[19] *See*, *e.g.*, I. Marrocco et al., Measurement and Clinical Significance of Biomarkers of Oxidative Stress in Humans, OXIDATIVE MEDICINE AND CELLULAR LONGEVITY (2017) [Tab 15]; S. Rose et al., Evidence of Oxidative Damage and Inflammation Associated with Low Glutathione Redox Status in the Autism Brain, 2 TRANSLATIONAL PSYCHIATRY (2012) [Tab 16].

[20] FreSHtracer binds to reduced forms of glutathione (GSH), but not to oxidized forms (GSSG). *See* E.M. Jeong, J.W. Shin, et al., Monitoring Glutathione Dynamics and Heterogeneity in Living Stem Cells, 12 INT. J. STEM CELLS, 367-379 (2019) [Tab 17].

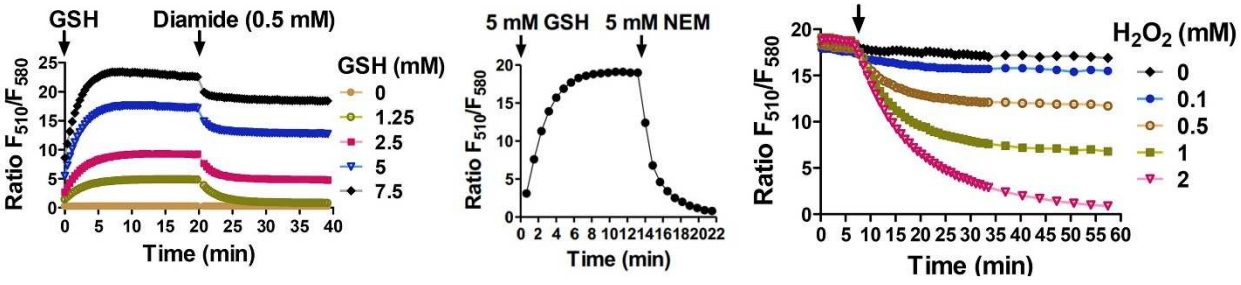
[21] As noted previously, GSH exists in significantly higher concentrations than protein thiols in cells. However, in experiments where cells are treated with high concentrations of cell-permeable thiol compounds, these compounds may compete with GSH in binding with the FreSHtracer. In these circumstances, experiments should be designed so that the protein thiols have a negligible effect on the FreSHtracer probe and the probe is able to properly monitor GSH levels.
See Jeong, Shin, et al., *supra* note 20 [Tab 17].

Fig. 1. FreSHtracer and its fluorescence spectral changes when it reacts with GSH



FreSHtracer has been tested in various living cell types and has been demonstrated to be highly effective at providing real-time measurements of intracellular GSH levels. The cell types tested include HeLa cells, RAW264.7 cells, human bone marrow-derived mesenchymal stem cells (hBM-MSCs), human embryonic stem cell-derived MSCs (hES-MSCs) and human umbilical cord-derived MSCs (hUC-MSCs). The use of FreSHtracer in these cells also did not cause any cytotoxic effects (*see infra*, note 27). Moreover, FreSHtracer can be removed from cells by washing and culturing them in a new medium. Experiments performed by Cell2in in living cells revealed that GSH levels are heterogeneous not only amongst different cells, but also amongst different cellular components, existing in different levels in the cytoplasm, nucleus, nucleolus and peripheral cytoplasm. Experiments have also confirmed that oxidative stress can produce dynamic changes in intracellular GSH levels. When ROS was increased, GSH concentration rapidly decreased and then remained unchanged before climbing up slowly, ultimately reaching its initial level.

Fig. 2. FreSHtracer Is a Reversible and Ratiometric Probe for Glutathione



2. In Vitro Studies Using FreSHtracer Show That High Glutathione Levels Correlate With Cells' Antioxidant State and Functionality

In the experiments summarized below, FreSHtracer was used to sort cells based on FR values or intracellular GSH concentrations. The experiments confirmed that low GSH levels correlate with aging and cellular senescence, while high GSH levels correlate with enhanced anti-aging activity and consequently, increased functionality. These results indicate that real-time GSH monitoring assays, such as FreSHtracer, can be used to sort and differentiate 'younger' cells with high GSH levels, high anti-oxidant capacity and high functionality from

'older' cells (*e.g.*, cells derived from high passage cell lines) that exhibit low GSH levels and are likely to show reduced functionality. Detailed results and accompanying figures for each of these experiments are available in Cell2in's Patent Cooperation Treaty applications, PCT/KR2018/014815 (Tab 28) and PCT/KR2018/014825 (Tab 29).

- *Human dermal fibroblasts (HDF)*

HDFs at their 32nd passage contained 44% less GSH than cells at their 6th passage. Fluorescence-activated cell sorting (FACS) was used to sort HDFs into two groups: GSH^{high} (top 0.2-30.2% of cells) and GSH^{low} (bottom 0.2-30.2% of cells) cells, as determined by FR values. GSH^{low} cells were 1.5 times larger in size compared to GSH^{high} cells and exhibited higher intracellular ROS levels, which was confirmed by treatment with dihydrorhodamine 123, a non-fluorescent ROS indicator. These findings are in accordance with previous studies that have demonstrated that cells become larger as they age, due to the increase in both number and size of lysosomes and aggregation of glycogen.[22] GSH^{low} cells also contained increased amounts of lipofuscin, which are primarily aggregates of oxidized proteins and considered a hallmark of aging.[23] Finally, GSH^{low} cells exhibited lower ki67 mRNA expression levels (proliferation marker), but higher expression levels for p21 (cell cycle inhibitor) and senescence-associated secretory phenotype (SASP)-related genes such as IL-1A and IL-1B. These results are consistent with previous experiments that have shown that increased ROS can lead to senescence, a stress response characterized by cells' inability to proliferate, morphological transformations, chromatin reorganization, altered gene expression and secretion of pro-inflammatory factors called SASP factors.[24] Cellular senescence ultimately leads to aging as well as development of age-related diseases mentioned above.

- *Human monocyte-derived dendritic cells*

Flow cytometry was used to sort dendritic cells based on GSH values into GSH^{high} (top 0.2-30.2% of cells), GSH^{mid} (top 30.2-62.5%) and GSH^{low} (bottom 0.3-32.7%) cells. Regardless of the cells' maturation, GSH concentration correlated with the cells' expression level of CD80, a surface protein that plays a critical role in T-lymphocyte activation. CD80 is a molecule expressed transiently on dendritic cells, activated B cells and macrophages and is known for its role in T-cell activation.[25] High GSH concentration therefore was shown to correlate with immune activity of dendritic cells.

- *Mouse T lymphocytes*

Mouse T lymphocytes were activated using CD3 and CD28 antibodies and then sorted into three groups based on GSH concentrations. Real-time quantitative PCR revealed mRNA levels of *foxp3* to be approximately 4-fold higher in GSH^{low} cells compared to GSH^{high} and

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[22] E. Robbins et al., Morphologic Changes Accompanying Senescence of Cultured Human Diploid Cells, 131 J. EXP. MED. 1211 (1970) [Tab 18].

[23] E.A. Georgakopoulou et al., Specific Lipofuscin Staining as a Novel Biomarker to Detect Replicative and Stress-Induced Senescence. A Method Applicable in Cryo-Preserved and Archival Tissues, 5 AGING, 37-50 (2013) [Tab 19].

[24] *See* N. Herranz and J. Gil, Mechanisms and Functions of Cellular Senescence, 128 J. CLIN. INVEST., 1238-46 (2018) [Tab 20]; A. Pole et al., Oxidative Stress, Cellular Senescence and Ageing, 3 AIMS MOLECULAR SCIENCE, 300-324 (2016) [Tab 21]; CELL SIGNALING TECHNOLOGY, Cellular Senescence (Last visited Sept. 4, 2019) [Tab 22].

[25] *See*, *e.g.*, S.V. Schmidt et al., Regulatory Dendritic Cells: There is More Than Just Immune Activation, 3 FRONT IMMUNOL. (2012) [Tab 23]; M.A., Concept of Reverse Costimulation and Its Role in Diseases, Chapter 2 (Developing Costimulatory Molecules for Immunotherapy of Diseases, 2015) [Tab 24].

GSH^{mid} cells. Foxp3 is a transcription factor acting as a critical regulator of regulatory T (Treg) cell development, function and differentiation.[26]

3. In Vitro Studies Using FreSHtracer Reveal That High Glutathione Levels Correlate With Increased Stem Cell Function

Importantly, for the purposes of this Citizen Petition, the use of FreSHtracer in various types of stem cells revealed that high GSH levels correlate with increased core stem cell properties such as the ability to self-renew, proliferate, migrate and differentiate. Experiments were conducted in vitro in hBM-MSCs, hES-MSCs, hUC-MSCs and murine embryonic stem. The results for each of these experiments, published in a peer-reviewed article, are briefly outlined below.

- *hBM-MSCs*[27]

Flow cytometry was used to sort hBM-MSCs based on FR values into FR^{high}, FR^{mid} and FR^{low} cells. As noted above, FR refers to the fluorescence ratio of F_{510} / F_{580}, which was shown to directly correlate with the GSH concentration. A higher FR value therefore indicates greater GSH concentration. FR^{high} stem cells displayed significantly enhanced cellular functions as demonstrated by CFU-F and chemoattraction to platelet-derived growth factors.

- *hES-MSCs*[28]

FACS was used to sort hES-MSCs based on FR values into FR^{high}, FR^{mid} and FR^{low} cells. The three groups showed no significant differences in proliferation rate. When cells were cultured in chondrogenic, adipogenic, or osteogenic induction media, FR^{high} and FR^{low} cells exhibited similar capacities to differentiate into chondrogenic, adipogenic and osteogenic lineages.

FR^{high} cells, however, showed significantly increased induction of certain lineage markers, including *SOX9*, *AP2* and *OCN*, compared to FR^{low} cells. FR^{high} cells also gave rise to approximately 4.7- and 4.9- fold higher numbers of CFU-F than FR^{mid} and FR^{low} cells, respectively. When individual CFU colonies were harvested and replated for a limiting dilution assay, CFU colonies from FR^{high} cells exhibited two-times enhanced clonogenic activity than FR^{low} cells. FR^{high} cells also showed greater chemoattraction to stromal derived factor 1 than FR^{low} cells. Finally, qPCR revealed that mRNA expression of pluripotency and migration-related genes such as *OCT4*, *SOX2*, *CXCR4, cMET*, *PDGFRA, PDGFRB, VEGFR1 and VEGFR2* was greater in FR^{high} cells than in FR^{low} cells.

- *hUC-MSCs*[29]

Flow cytometry was used to sort hUC-MSCs into FR^{high} and FR^{low} cells. CFU-F assay revealed greater colony formations for FR^{high} cells than FR^{low}.

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[26] *See*, *e.g.*, W. Lee and G.R. Lee, Transcriptional Regulation and Development of Regulatory T Cells, 50 EXPERIMENTAL & MOLECULAR MEDICINE (2018) [Tab 25]; X. Tai et al., Foxp3 Transcription Factor is Proapoptotic and Lethal to Developing Regulatory T Cells Unless Counterbalanced by Cytokine Survival Signals, 38 IMMUNITY, 1116-28 (2013) [Tab 26].
[27] *See* E.M. Jeong, J.H. Yoon et al., Real-Time Monitoring of Glutathione in Living Cells Reveals that High Glutathione Levels are Required to Maintain Stem Cell Function, 10 STEM CELL REPORTS, 600-614 (2018) [Tab 27].
[28] *Id.* [Tab 27]
[29] *See* Jeong, Shin et al., *supra* note 20 [Tab 17].

- *Murine embryonic stem cells*[30]

FACS was used to sort hUC-MSCs into FR^{high}, FR^{mid} and FR^{low} cells. Limiting dilution assay revealed FR^{high} cells exhibited significantly enhanced clonogenic capacity and therefore self-renewal activity than FR^{low} cells. FR^{high} cells also showed greater mRNA expression of pluripotency-related genes such as *Nanog*, *Klf2*, *Klf4*, *Rex1* and *Esrrb*. When the cells were differentiated by forming embryoid bodies (EBs), FR^{low} cells exhibited defective differentiation capacity compared to FR^{high} cells, as evidenced by reduced EB formation and lower induction of lineage markers such as *Neurog2*, *Olig2*, *T* and *Nkx2.5*. This was further validated by *in vitro* neuronal differentiation of the cells, which revealed reduced amount of βIII-tubulin^{+} neurons and impaired induction of several neuronal markers such as *Neurog1*, *Neurod1*, *Ascl2*, and *Gfap* in FR^{low} cells compared to FR^{high} cells.

4. In Vivo Studies Using FreSHtracer Reveals That High Glutathione Levels Improve Stem Cell Therapeutic Efficacy

The use of FreSHtracer in various in vivo disease models further corroborated the in vitro findings above. Experiments using a mouse asthma model and rat osteoarthritis model confirmed that higher GSH levels correlate with enhanced stem cell function and improved therapeutic efficacy. These results also confirmed the benefits of real-time GSH monitoring methods such as FreSHtracer.

- *Mouse asthma model*[31]

The therapeutic effectiveness of hES-MSCs with different FR ratios was tested in a mouse model of virus-associated asthma. Allergic sensitization in the mice was generated with ovalubin (OVA) and poly(I:C). Histological examinations showed reduced inflammation around the bronchial and perivascular areas in the lungs of FR^{high} cell-injected mice, compared to FR^{low} cell-injected mice. Fewer inflammatory cells were also found in the bronchoalveolar lavage fluid of FR^{high} cell-injected mice. Tumor necrosis factor α and interleukin-17 levels were lower, while IL-10 levels were higher in the bronchoalveolar lavage fluid of FR^{high} cells. qPCR revealed decreased mRNA expression of inflammatory cytokines in the lung tissues of FR^{high} cell-injected mice. Immunohistochemical staining with human β_2-microglobulin antibody showed a significant increase in engrafted cells, identified as type-2 alveolar cells, in the lungs of FR^{high} cell-injected mice. Thus, the results confirmed that the injected stem cells had migrated and differentiated into the alveolar epithelium and contributed to tissue generation.

- *Rat osteoarthritis model*[32]

The therapeutic effectiveness of hES-MSCs with different GSH levels was tested in an osteoarthritis rat model. Osteoarthritis-induced rats' joints were prepared by rupturing the anterior cruciate ligament (ACL). Rats that received transplantation of stem cells with higher levels of GSH had significantly heightened cartilage regeneration efficacy. Stem cells with higher levels of GSH exhibited greater expression of components of cartilage, including glycosaminoglycan (GAG) and Type II collagen.

In conclusion, these results suggest that GSH concentration not only correlates with cells' antioxidation state and functionality, but also with the ability to self-renew, proliferate and

[30] *See* Jeong, Yoon et al., *supra* note 27 [Tab 27].
[31] *See id.* [Tab 27].
[32] *See* PCT/KR2018/014815 11/28/2018, Method of Improving Quality of Therapeutic Cells Through Real-Time Glutathione Monitoring [Tab 28].

differentiate. GSH levels can therefore be used as a biomarker to evaluate cells' antioxidant capacity and stemness, providing real-time feedback on their quality.

The next section of this Citizen Petition will provide an overview of FDA requirements regarding potency assays before discussing further the promising application these findings offer: a new potency assay for cellular therapies, including stem cell therapies, that can monitor and control cells for relevant functional cell characteristics that contribute to the products' clinical effectiveness.

C. Regulatory Framework: Potency Assays For Cellular Therapies

1. General Guidance From The Agency

Potency is a critical quality attribute of any biological product and is defined by FDA regulations as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result."[33] Under the PHS Act, all biological products, including stem cells and cell therapeutics, must be "safe, pure, and potent" to receive FDA marketing approval.[34] Sponsors of biological products must demonstrate such properties of their products by including in their BLA, data derived from nonclinical and clinical studies demonstrating that the products subject to the applications meet prescribed requirements of safety, purity and potency.[35]

FDA regulations establish that no lot of any licensed biological product may be released without conformance testing, which includes tests for potency.[36] Any potency assay must be validated before the product is licensed.[37] Many biological products undergo changes in their manufacturing process, including site transfer and scale-up, during development and post-marketing approval. In these circumstances, potency tests allow comparability studies, enabling manufacturers and the Agency to compare products manufactured under different conditions. Finally, potency tests are performed as part of stability testing to confirm that the biological products stay within their pre-defined parameters during their intended period of use. For these reasons, potency tests help ensure that consistently manufactured products are being produced and administered throughout product development as well as the entire product life cycle.

The Agency's regulations do not identify specific potency assays required to support BLA submissions. Instead, the regulations are drafted in general terms allowing potency assays to be "in vitro or in vivo tests, or both" and specify only that they be "specifically designed for each product so as to indicate its potency in a manner adequate to satisfy the interpretation of potency given by the definition in § 600.3(s)."[38]

Over time, FDA has issued several guidances that further set forth the Agency's thinking and recommendations on potency assays. The Guidance for FDA Reviewers and Sponsors, Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs) (hereinafter, the "CMC

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[33] 21 C.F.R. § 600.3(s).
[34] The PHS Act, 42 U.S.C. § 262(B) ("The Secretary shall approve a biologics license application (i) on the basis of a demonstration that (I) the biological product that is the subject of the application is safe, pure, and potent").
[35] 21 C.F.R. § 601.2(a).
[36] *Id.* §§ 601.20(a) and 610.1.
[37] *Id.* § 211.165(e); *See* CMC Guidance, *infra* note 39, at 20.
[38] 21 C.F.R. §§ 211.165(e) and 610.10.

Guidance"),[39] published in 2008, directs sponsors to ensure that their INDs include sufficient information to demonstrate the proper identification, quality, purity and potency of the cells. Therein, FDA recommends product testing, which includes assessment of the product's potency, "throughout the manufacturing process, including on the manufacture of cell banks" to evaluate the manufacturing process itself and to ensure the quality and consistency of the product. The CMC Guidance directs sponsors to describe in their INDs all assays used to measure potency. The CMC Guidance recommends that the potency assays be quantitative and measure a biological activity, but if that is not feasible, a quantitative physical assay that correlates with and is used in conjunction with a qualitative biological assay is also allowed.[40] Proposed specifications for potency assays should also be included in the INDs, preferably in a table format.[41] The CMC Guidance also directs FDA reviewers to document testing performed and the appropriateness of that testing.

FDA has also issued a more specific guidance on potency assays. The 2011 Guidance for Industry, Potency Tests for Cellular and Gene Therapy Products (hereinafter, the "Potency Tests for CGT Products Guidance")[42] further outlines the types of potency tests that could support an IND or a BLA of cellular and gene therapy products that fall under section 351 of the PHS Act. The guidance first notes that while evidence of clinical effectiveness is largely demonstrated through adequate and well-controlled clinical trials, oftentimes the use of clinical study data is not a practicable method to assess potency for a lot release. This is because the clinical data cannot be made available prior to release of individual product lots or cannot be coupled to individual lots.[43] As such, the guidance recommends three other approaches to measuring potency.

The guidance makes clear that an ideal potency assay reflects the biological product's mechanism of action or intended biological effect.[44] Accordingly, the most preferred method is a biological assay or a bioassay performed in a living system that measures the product's biological activity directly. Bioassays can be in vitro studies in organ, tissue or cell culture systems or in vivo animal studies.

In cases where the development of a suitable bioassay is not possible, FDA permits a non-biological analytical assay. According to the guidance, such assays would be performed outside of the living system and use a surrogate measurement that correlates to the product's biological activity. The correlative relationship would, however, need to be substantiated. The guidance allows for various approaches to doing so, including "comparison to preclinical/proof of concept data, in vivo data (animal or clinical), or in vitro cellular or biochemical data."[45] The adequacy of data used to demonstrate the correlation is evaluated on a case-by-case basis and depends on factors such as type and relevance of the correlation being made, the amount of information accumulated, the degree of understanding of the biological activity of the product, and the degree to which the surrogate measurement reflects biological activity. FDA makes clear that, although analytical assays are not bioassays, they can still provide extensive product characterization data by evaluating immunochemical (*e.g.*, quantitative flow cytometry, enzyme-

[39] FDA, Guidance for FDA Reviewers and Sponsors, Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs) (Apr. 2008), https://www.fda.gov/media/73624/download.
[40] CMC Guidance, at 19.
[41] CMC Guidance, at 20.
[42] FDA, Guidance for Industry, Potency Tests for Cellular and Gene Therapy Products (Jan. 2011), https://www.fda.gov/media/79856/download.
[43] Potency Tests for CGT Products Guidance, at 5
[44] Potency Tests for CGT Products Guidance, at 6.
[45] Potency Tests for CGT Products Guidance, at 8-9.

linked immunosorbent assay), molecular (*e.g.*, reverse transcription polymerase chain reaction, quantitative polymerase chain reaction, microarray) or biochemical (*e.g.*, protein binding, enzymatic reactions) attributes of the product.[46]

Finally, in cases where a single bioassay or an analytical assay cannot constitute an acceptable potency assay, the Agency allows a matrix approach. Here, the idea is that the use of multiple complementary assays and their combined results would provide an acceptable measure of potency. This is because the active ingredient of stem cell therapies tends to be whole cells, and it is often impossible to assess the activity of these cells using only one attribute or characteristic of a cell.

Because potency assays are designed specifically for each product, the Agency has long maintained its position of allowing sponsors considerable freedom to design and choose potency assays that would fulfill the Agency's requirements.[47] However, there are certain biologics and CGMP regulations that all potency assays used for release testing of licensed biological products must comply with:

1. Indicate potency (biological activity or activities) specific to the product (21 CFR 600.3(s) and 610.10; and 21 CFR 210.3(b)(16)(ii));
2. Provide test results for release of the product (21 CFR 610.1; 21 CFR 211.165(a));
3. Provide quantitative data (21 CFR 211.194; *see also* 21 CFR 600.3(kk); 21 CFR 211.165(d); 211.165(e));
4. Meet pre-defined acceptance and/or rejection criteria (21 CFR 211.165(d); *see also* 21 CFR 600.3(kk); and 21 CFR 210.3(b)(20));
5. Include appropriate reference materials, standards, and/or controls (*see* 21 CFR 210.3(b)(16)(ii) and 211.160);
6. Establish and document the accuracy, sensitivity, specificity and reproducibility of the test methods employed through validation (21 CFR 211.165(e) and 211.194(a)(2));
7. Measure identity and strength (activity) of all active ingredients (21 CFR 211.165(a); *see also* 21 CFR 210.3(b)(7));
8. Provide data to establish dating periods (*see* 21 CFR 600.3(l) and 610.53(a)); and
9. Meet labeling requirements (21 CFR 610.61(g)(3) and 610.61(r))

Because cellular therapies are complex and highly variable, there are several challenges to designing meaningful as well as relevant potency assays. The Potency Tests for CGT Products Guidance also lists some examples of these hurdles.[48] For example, the inherent variability of cellular products can be problematic and be hard to control from one lot to another. Cellular products often involve multiple active ingredients and/or multiple mechanisms of action, which may be complex and/or not have been fully characterized. Appropriate reference standards are also difficult to develop.

Finally, because the development of potency assays can be a challenging and time-consuming endeavor and potency assays are critical to determining the biological activity of the product, the guidance recommends development of potency assays from "during early product

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[46] Potency Tests for CGT Products Guidance, at 7.
[47] Potency Tests for CGT Products Guidance, at 1 and 3; Transcript of FDA, CBER Meeting of Cellular, Tissue and Gene Therapies Advisory Committee, Gaithersburg Hilton, Gaithersburg, MD, at 15 (Feb. 9, 2006), https://wayback.archive-it.org/7993/20170404052015/https://www.fda.gov/ohrms/dockets/ac/06/transcripts/2006-4205T1.pdf ("The Office of Cellular, Tissue and Gene Therapy has no particular preference for any type of assay. The acceptability of all potency assays will be determined on a case by case basis.").
[48] The Potency Tests for CGT Products Guidance, at 4-5.

development."[49] Early potency assay development has a number of advantages, including demonstrating product activity, quality and consistency throughout product development, providing data to establish specifications for lot release, providing a basis for assessing manufacturing changes, and evaluating product stability.[50]

2. Guidance Regarding Specific Assays

FDA has in the past recommended the use of specific assays to assess potency for certain categories of biological products. Specifically, the Agency's 2014 HPC, Cord Blood Guidance includes detailed recommendations for potency assays. The recommendations are directed to a specific type of stem cell therapy, more commonly known as hematopoietic progenitor cell (HPC), cord blood, currently the only FDA-approved stem cell-based products.[51] Their active ingredient consists of HPCs that express the cell surface marker CD34.[52] After formulation, the cord blood product is generally cryopreserved and stored for several years before it is administered to a patient.

The guidance establishes that a sponsor of a HPC, Cord Blood product may satisfy the requirements for purity and potency assays by performing the combination of three assays below on pre-cryopreservation samples.[53] For each assay, the guidance also sets forth acceptance criteria to which the final products need to conform to in order to be released for clinical use[54]:

1. Total nucleated cell (TNC) count
    a. Release Criteria: The total number of nucleated cells in the HPC, Cord Blood should be adequate to provide, after thawing, at least 1.7 x 10^7 nucleated cells/kg of body weight of the prospective recipient. Because the weight of the prospective recipient is unknown at the time of storage, the manufacturer should store HPC, Cord Blood units that contain at least 5.0 x 10^8 total nucleated cells per product.

2. Viable nucleated cells
    a. Release Criteria: The sponsor should demonstrate by a validated assay that at least 85% of the nucleated cells in the HPC, Cord Blood unit are viable after volume reduction and before cryopreservation.

3. Viable CD34+ cells
    a. Release Criteria: ≥ 1.25 x 10^6 viable CD34+ cells/unit HPC, Cord Blood. The percentage of viable nucleated cells expressing the hematopoietic progenitor cell marker CD34+ in a normal HPC, Cord Blood unit should be at least 0.25% of the

[49] The Potency Tests for CGT Products Guidance, at 9.
[50] *Id.*
[51] FDA, FDA Warns About Stem Cell Therapies (Last Updated Nov. 16, 2017), https://www.fda.gov/consumers/consumer-updates/fda-warns-about-stem-cell-therapies; See also A. Taylor, "FDA Cracks Down on Purveyors of Stem Cell Treatments", THE SCIENTIST (Dec. 21, 2018) [Tab 30]; and W. Wan and L. McGinley, "Miraculous Stem Cell Therapy Has Sickened People in Five States", WASHINGTON POST, (Feb. 27, 2019) [Tab 31].
[52] See, *e.g.*, M.D. Anderson Cancer Center's HPC, Cord Blood package insert at https://www.fda.gov/media/114119/download.
[53] The guidance also allows other assays to be submitted as part of BLAs as long as sponsors provide evidence demonstrating that their modifications or deviations provide similar assurances of safety, purity, potency of their product. *See* Potency Tests for CGT Products Guidance, at 11.
[54] HPC, Cord Blood Guidance at 11 and 38.

total viable nucleated cell content after volume reduction and before cryopreservation.

There are currently eight HPC, Cord Blood products approved by FDA.[55] A review of the publicly available portions of their BLAs and other materials available on the FDA website reveals the three analytical assays above being used as the primary potency assays.[56] For example, according to the package insert of the most recently approved product sponsored by M.D. Anderson Cord Blood Bank, "The potency of cord blood is determined by measuring the numbers of total nucleated cells (TNC) and CD34+ cells, and cell viability. Each unit of HPC, Cord Blood contains a minimum of 9.0 x 10^8 total nucleated cells with at least 1.25 x 10^6 viable CD34+ cells at the time of cryopreservation." The CMC review of Cleveland Cord Blood Center's CLEVECORD states, "Total Nucleated Cell count (TNC) is the primary potency test, but viable CD34+ cell counts, TNC viability, and (b)(4) are also used as complementary tests for the evaluation of the quality and potency of a HPC, Cord Blood product."

The products also usually employ a fourth additional purity and potency assay. As an example, M.D. Anderson Cancer Center's HPC, Cord Blood product performs colony assays for CFU-GM, CFU-GEMM and BFU-E on the final product to be infused, with growth as the release criterion.[57]

D. Real-Time GSH Monitoring Assays Meet FDA Criteria For Potency Assays and Should Be Relied Upon By Sponsors And FDA

1. Real-Time GSH Assays Are Non-Biological Analytical Assays Using Intracellular GSH Concentration as a Surrogate Marker for Antioxidant Capacity and Stemness

Cell2in believes real-time GSH monitoring assays meet FDA's requirements for potency assays for cell therapy products. The assays are non-biological analytical assays, discussed in Section III.B.2 of the Potency Tests for CGT Products Guidance, and can be used in conjunction with an already existing bioassay or in circumstances where development of a suitable bioassay for a cellular therapy is not possible. The GSH assays are able to provide extensive product characterization data, including information on the cellular therapeutic product's ability to withstand ROS-induced cellular damage. In stem cells, the assays

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[55] The list of approved stem cell products is available at https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products.

[56] *See* package insert for M.D. Anderson Cancer Center's HPC, Cord Blood, *supra* note 52, at 9 ("The potency of cord blood is determined by measuring the numbers of total nucleated cells (TNC) and CD34+ cells, and cell viability. Each unit of HPC, Cord Blood contains a minimum of 9.0 x 10^8 total nucleated cells with at least 1.25 x 10^6 viable CD34+ cells at the time of cryopreservation."); package insert for Cleveland Cord Blood Center's HPC, Cord Blood, BLA 125594 at https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/clevecord-hpc-cord-blood ("The potency of cord blood is determined by measuring the numbers of total nucleated cells (TNC) and CD34+ cells, and cell viability."); CMC Review of Original Submission BLA 125594, at 38 ("Total Nucleated Cell count (TNC) is the primary potency test, but viable CD34+ cell counts, TNC viability, and (b)(4) are also used as complementary tests for the evaluation of the quality and potency of a HPC, Cord Blood product."); and package insert for Bloodworks' HPC, Cord Blood, BLA 125585 at https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/hpc-cord-blood-bloodworks ("The potency of the cord blood is determined by measuring the numbers of total nucleated cells (TNC) and CD34+ cells, and cell viability.").

[57] *See* M.D. Anderson Cancer Center package insert, *supra* note 52, at 21; CMC Review of Original Submission BLA 125657, at 11 and 80, https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/hpc-cord-blood-md-anderson-cord-blood-bank.

demonstrate the cells' stemness or ability to self-renew, migrate and differentiate, all of which are important requisites for any potent stem cell therapy.

As discussed above, a non-biological analytical assay may be used to demonstrate potency if the surrogate measurement can be identified and its correlation to a relevant product-specific biological activity, specified.[58] GSH assays use intracellular GSH concentration as a surrogate measurement of cells' antioxidant capacity and the stem cells' biological activity. As discussed above in Sections II.B.3 and 4, using FreSHtracer, Cell2in has provided strong evidence supporting the finding that higher GSH levels can be used as a proxy for greater stemness properties. Greater levels of intracellular GSH, indicated by greater FR, correlated with enhanced ability to self-renew (as demonstrated by the limiting dilution assay), migrate (as demonstrated by chemotaxis assay), proliferate and differentiate (as demonstrated by the CFU assay). Stem cells with greater GSH levels also exhibited greater mRNA expression of pluripotency and migration-related genes. These results were shown in four different types of stem cells. Studies in vivo also confirmed that intracellular GSH level correlates with therapeutic effectiveness, as shown in a mouse model of asthma and rat osteoarthritis model.

Cell2in believes that real-time GSH monitoring assays can also meet, or will be able to meet with further development and validation, the additional requirements for potency assays outlined in the Potency Tests for CGT Products Guidance. Accordingly, the use of these assays should be encouraged by the Agency for the development and commercialization of stem cell therapy products.

2. Real-Time GSH Monitoring Assays Should Be Used As Potency Assays For HPC, Cord Blood Products

Cell2in also believes that real-time GSH monitoring assays should be used as potency assays for HPC, Cord Blood products in addition to cellular therapies in general. As discussed above in Section II.C.2 of this Citizen Petition, FDA enables HPC, Cord Blood Products to meet their potency requirements by performing three assays – TNC, viable nucleated cells, and viable CD34+ cells – and demonstrating that the final products meet the pre-established criteria. Although the Agency allows for the use of potency assays other than these to satisfy the requirements, none of the currently approved HPC, Cord Blood products seem to have opted for this path, and all such eight products primarily rely on these three assays to substantiate their potency.

As the Agency is likely aware, there is much controversy as to whether these three assays provide an adequate measure of the products' potency. As an initial matter, TNC and viable nucleated cell count methods include both stem cells and other types of cells.[59] The CD34 marker is also not exclusive to stem cells. In sum, although these three parameters provide some information on cellular viability and count, they do not provide accurate quantification of the active stem cells.

In addition, cellular viability and count, while a contributing factor to cord blood products' potency, are not indicative of their biological activity, which is the most significant feature of potency. One peer-reviewed publication describes the three parameters to be "woefully inadequate" as cell product potency markers in the absence of data from bioassays that reflect

[58] Potency Tests for CGT Products Guidance, at 8-9.
[59] CRYOCELL INTERNATIONAL, Counting Your Baby's Cord Blood Stem Cells: What Do TNC, CD34+, CFU and Other Numbers Mean? (Jan. 24, 2019) [Tab 32].

the mechanism of action.[60] As one of the comments submitted for the draft version of the HPC, Cord Blood Guidance also noted, a cord blood product with high cellular count and viability does not necessarily mean successful engraftment, for a "UCB [umbilical cord blood] unit of low volume and cell number might have a high proportion of primitive stem cells, while a UCB unit with a high cell number may consist primarily of mature stem cells on the verge of becoming lineage-specific cells and exhibiting such a low proliferation capability that the unit might not even demonstrate short-term engraftment."[61]

FDA's Cellular, Tissue and Gene Therapies Advisory Committee voiced the similar concerns during its 2007 meeting on the HPC, Cord Blood Guidance. Committee members stated that the "ultimate potency assay is whether or not that product engrafts in the patient."[62] While there may be confounding factors that can affect engraftment such as the stage of patients' disease or existence of post-transplant infections, ultimately, the indisputable fact is that the counts of TNC, viable nucleated cell and viable CD34+ cells cannot accurately predict engraftment.[63] A truly meaningful potency assay for stem cell therapies should measure stemness – the cells' ability to self-renew, proliferate and differentiate.

Although the CFU assay is not among the three potency assays recommended in the HPC, Cord Blood Guidance, it is one of the most common assays performed on stem cells, including HPC, Cord Blood products. The CFU assay uses a stem cells' ability to proliferate as a proxy for potency. However, the inter-laboratory reproducibility of the assay is poor, which has prevented its wide acceptance.[64] The inability to standardize and reproduce the assay was also noted during the Committee's meeting mentioned above.[65] Moreover, while the CFU assay provides confirmation that the stem cells have the ability to proliferate, it can be difficult to quantify this ability accurately, especially if counts are made manually, as they frequently are.[66]

Another drawback is its long incubation period. The cells need to be incubated for at least 7-14 days before the colonies can be counted, making the assay an impractical choice for a lot release potency assay. Time constraints for release testing is a significant and oft-cited challenge, for many cellular products need to be administered within hours of harvest or formulation and potency assays ideally should be performed on the product immediately before administration. However, many currently existing assays such as the CFU assay take a considerable amount of time to perform and obtain results.

Given the shortcomings in the assays currently identified in FDA's HPC, Cord Blood Guidance and the commonly used CFU assay, there is a need for a potency assay that reflects how stem cell therapies work: the cells' migration to site of injury, proliferation and self-renewal,

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[60] J. Basu and J. Ludlow, Cell-based Therapeutic Products: Potency Assay Development and Application, REGENERATIVE MEDICINE, 9(4), 497-512 (2014) [Tab 33].
[61] HemoGenix Inc., Comment to Docket FDA-2008-D-0520 [Tab 34].
[62] Transcript of FDA, CBER Meeting of Cellular, Tissue and Gene Therapies Advisory Committee, Gaithersburg Hilton, Gaithersburg, MD, at 168-69 (Mar. 30, 2007), https://wayback.archive-it.org/7993/20170404045549/https:/www.fda.gov/ohrms/dockets/ac/07/transcripts/2007-4291T2.pdf.
[63] *Id.* at 168.
[64] *See*, *e.g.*, R. Amarandi et al., Advantages of Graphene Biosensors for Human Stem Cell Therapy Potency Assays, BIOMED RES INT. (2018) [Tab 35]; D. Pamphilon et al., Current Practices and Prospects for Standardization of the Hematopoietic Colony-Forming-Unit (CFU) Assay: A Report by the Cellular Therapy Team of the Biomedical Excellence for Safer Transfusion (BEST) Collaborative, 15 CYTOTHERAPY, (2013) [Tab 36].
[65] *See* Transcript of FDA, CBER Meeting of Cellular, Tissue and Gene Therapies Advisory Committee (Mar. 30, 2007), *supra* note 62.
[66] Powell et al., Variability in Subjective Review of Umbilical Cord Blood Colony Forming Unit Assay, 90 CYTOMETRY PART B: CLIN CYTOMETRY, 517-524 (2016) [Tab 37]; Pamphilon et al., *supra* note 64 [Tab 36].

which is followed by differentiation into the correct cells and reconstitution of the appropriate tissue or organ. Real-time GSH monitoring assays, including the FreSHtracer, address this need by using intracellular GSH levels as an indicator of these exact functional characteristics, or stemness. Such GSH assays have advantages over the TNC assay, viable nucleated cell assay, viable CD34+ cell assay, and the CFU assay, and at a minimum, should be used in conjunction with these assays. Moreover, real-time GSH monitoring assays can be used through various phases of product development from early stages of research to maintain cell line quality and during the manufacturing process as part of in process testing to ensure the potency and quality of the product. GSH assays can also be performed on the final product for lot release and rejections.

E. Conclusion

Cell2in applauds FDA's issuance of guidances that have clarified and provided further details on the statutory and regulatory requirements for potency assays. However, these guidances no longer reflect the state-of-the-art technology available to accurately assess potency. New technologies, such as real-time monitoring of GSH levels, provide researchers with powerful new tools to characterize cellular therapies. The Agency should update its guidance documents to reflect the current state of the art on this important issue. Specifically, Cell2in requests that the Agency update its CMC Guidance, Potency Tests for CGT Products Guidance, and/or HPC, Cord Blood Guidance to identify real-time GSH monitoring as one means of measuring potency for purposes of submitting a BLA for cellular therapies, including stem cell therapies, under Section 351 of the PHS Act. We also invite dialogue with FDA regarding possible efforts to standardize, validate and further develop such GSH assays, including FreSHtracer, for widespread acceptance and use as a potency assay.

III. ENVIRONMENTAL IMPACT

This Citizen Petition qualifies for a categorical exemption from the requirement to submit an environmental assessment under 21 C.F.R. §§ 25.30 and/or 25.31.

IV. ECONOMIC IMPACT

Information regarding economic impact will be submitted upon FDA's request following review of this Citizen Petition pursuant to 21 C.F.R. § 10.30(b).

V. CERTIFICATION

The undersigned certifies that, to the best knowledge and belief of his knowledge, this Citizen Petition includes all information and views on which the petition relies, and that it includes representative data and information known to the petitioner that are unfavorable to the petition.

Respectfully submitted,

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