Stem Cell Activation by Natural Products

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Abstract

Bone marrow stem cell therapies, based on transplant of hematopoietic stem cells (HSCs) have been used for over 50 years in the treatment of blood disorders including leukemia, lymphoma, and auto-immune disorders. Embryonic stem cells (ESCs) were discovered in the late 1990's by Evans, Kauffman, and Martin. J Thompson developed procedures to isolate and expand ESCs from human embryos. Since ESCs could differentiate into any cell in the body, this expanded application of stem therapy to include broad areas in disease treatment and regenerative medicine. Use of ESCs was also associated with ethical/religious issues since their derivation from an embryo also destroyed the embryo. Recent research has shown that ESCs may form tumors following transplantation while adult stem cells have gained significant support for safety and efficacy in the treatment of skeletomuscular disorders and several other indications. Detailed studies have shown no evidence for malignant tumor formation from adult stem cell transplants. There are limitations of adult stem cell transplants including cost, potential safety issues, and the necessity of autologous transplants. Biological products activating endogenous HSCs have been used clinically in the treatment of anemia (recombinant human erythropoietin) and immuno-suppression (recombinant human G-CSF), resulting from chemotherapy for over 20 years. Recently, the endogenous cytokine BMP11 aka GDF11 has been shown to activate adult stem cells including Satellite muscle stem cells and possibly neural stem cells (NSCs). Additional studies have indicated that combined inhibition of GSK3-beta and HDAC-I may induce activation of NSCs, MSCs, and possibly other adult stem cell populations. We have developed a series of stem cell functional and activation assays measuring proliferation, migration, and epigenetic reprogramming. We describe initial results and validation data in this report. We also show that a new series of nutraceutical products, NutraVivo™ Dietary Supplements (Brain Grow Activator, Brain Grow Stimulator, and Brain Grow Energizer), activate human stem cells by these assays and from the performance of stem cells derived from a patient taking these nutraceuticals. Cell-based assays of stem cell function have application to quality control testing and development of new drug candidates.

INTRODUCTION

Cell-based assays are a unique tool in biotechnology with application to high-throughput quantitative analysis of specific biological events and underlying molecular events. Individual genes and specific microRNA binding events may be quantified in a live human cell system reflecting *in vivo* physiological systems. Here we describe a series of assays that quantify various aspects of stem cell activation including proliferation, migration, and epigenetic reprogramming. Many *in-vivo* adult stem cells reside in quiescence within the stem cell niche and these cells are activated by various stimuli. For example, a wound to the skin results in elaboration of substance P from the site of injury. Substance P, a well-known neurotransmitter with multiple biological effects mobilizes MSC from bone marrow to migrate to the site of injury through complex interplay of chemokines and their receptors, e.g., the well-known interactions between SDF1α and CXCR4. Activated stem cells migrate to sites of inflammation and exert anti-inflammatory and other biological responses leading to tissue regeneration and wound healing. Activation of stem cells involves the migration, proliferation, and reprogramming of cells. Migration is the movement of cells from one location to another, proliferation is the cell expansion and growth, and reprogramming results in altered gene expression, either by increased or decreased gene expression due to altered DNA methylation patterns. While up-regulation of certain well-known genes such as the pluripotency gene, Oct 3/4 and Sirtuin I are known to be associated with stem cell activation, the epigenetics of stem cell activation are not yet completely understood. Activation of endogenous stem cells is known to occur during a variety of different processes including organogenesis and regeneration, wound healing, and inflammation. Based on a desire for natural stem cell activation, Vitro Biopharma scientists used stem cell-based assays to develop the novel products: Brain Grow™ Activator, Brain Grow™ Stimulator, and Brain Grow™ Energizer for the effective activation of adult human stem cells. These assays also improve investigation methods with high throughput technology that allows determination of the pharmacology and dose response characteristics of nutraceuticals, pharmaceuticals, and other stem cell activation agents as well.

MATERIALS AND METHODS

Cell Culture

Native human cord blood-derived MSCs (Vitro Biopharma Cat. No. SC00A1 were grown to 90% confluency in T-25 tissue cultured flasks (BD Falcon, Cat. No. 353108) in MSC-Gro™ low serum, complete medium (Vitro Biopharma Cat. No. SC00B1). Cells were detached using a collagenase formulation, Accutase (Innovative Cell Technologies Inc., Cat No. AT-104) using 37^{o}C incubation for 15 minutes and placed into a

15mL conical tube. Cells were collected via centrifugation (450 x g) for 7 minutes. Cell supernatant was aspirated off and cells were resuspended in 1mL PBS and counted on a Beckerman-Coulter Z2 particle counter (range 10 μm-30 μm).

Cell Migration Assay

Cell Culture

One million cells were resuspended in 10mL MSC-Gro™ serum free, quiescent medium (Vitro Biopharma Cat. No. SC00B17) containing 5μg/mL mitomycin C (Sigma, Cat. No. M4287) and incubated for 2hrs at room temperature with end-to-end agitation at 7 RPM. Cells were centrifuged (450 x g) for 7 minutes and washed out with PBS. Cells were resuspended in 1mL MSC-Gro™ low serum, complete medium (Vitro Biopharma Cat. No. SC00B1) and were plated at 25,000/well in black 96 well cell culture plates, TC-coated (Thermo Scientific, Cat. No. 165305) containing cell seed stoppers (Platypus, Cat. No. CMAUFL4) to form a cell free zone and placed in 5%CO_2, 1%O_2, 94% N_2 at 37°C in a humidified chamber for 24hrs.

CellTracker Green Staining and Activation Agent Dosing

Cells were washed once with PBS then incubated in serum free, MSC-Gro™ (Vitro Biopharma Cat. No. SC00B17) containing 5μM CellTracker Green CMFDA (Molecular Probes, Cat. No. C7025) at 37°C for 30 minutes. Cells were washed with serum free, MSC-Gro™ (Vitro Biopharma Cat. No. SC00B17) and incubated for 30 minutes at 37°C. Cells were washed once with PBS and replaced with MSC-Gro™ serum free, quiescent medium (Vitro Biopharma Cat. No. SC00B17) containing different concentrations of activating agents. Substance P was purchased from Tocris Bioscience, (Cat. No. 1156) and stem cell activators: Brain Grow Activator, Brain Grow Stimulator, and Brain Grow Energizer (NutraVivo™). A Top Seal (PerkinElmer, Cat. No. 6050195) covered the plate and it was placed in a BioTek Cytation3 Imaging Reader. Kinetic data was acquired every 2hrs for 24hrs using GFP data acquisition. The gas phase throughout the acquisition of kinetic data was 5% O_2, 5% CO_2 with the balance of nitrogen maintained by a BioTek CO_2/O_2 gas controller. Cellular analysis was performed using Gen5 software on the 2.5x images capture setting. This was done to detect the actual number of cells migrating or invading to the bottom of the membrane and ignore all other portions of the image. Gen5 software was used to determine the area of the detection zone of post-migration wells in comparison with controls to calculate percent closure using imaging data. Values were imported into Origin 8.1 and graphed.

Proliferation Assay

Cell Culture

100,000 cells were resuspended in 2mL MSC-Gro™ low serum, complete medium (Vitro Biopharma Cat. No. SC00B1) and plated at 5,000/well in tissue cultured black 96 well plates (ThermoScientific, Cat. No. 165305) and placed in 5%CO_2, 1%O_2, 94%N_2 at 37°C in a humidified chamber for 24hrs.

Cell Staining with PrestoBlue and Addition of Growth Factors/Activating Agents

Cells were washed 3x with PBS and replaced with MSC-Gro™ serum free, quiescent media (Vitro Biopharma Cat. No. SC00B17) containing different concentrations of stem cell activators: Brain Grow Activator, Brain Grow Stimulator, and Brain Grow Energizer (NutraVivo™). Cells were stained with 10μL PrestoBlue/well (Invitrogen, Cat. No. A13261) and incubated in the dark in a glove box at 1%O_2, 5%CO_2 at 37°C for 30 minutes. The plate was placed in a Turner Biosystems Modulus Microplate Reader and read using a FITC filter (Ex= 490nm, Em >510nm) for fluorescent intensities at Day 0 and Day 3.

Gene Expression Analysis

Cell Culture

Native human cord blood-derived MSCs (Vitro Biopharma Cat. No. SC00A1) were expanded from cryopreservation in a T-25 tissue cultured flasks (BD Falcon, Cat. No. 353108) in MSC-Gro™ low serum, complete medium (Vitro Biopharma Cat. No. SC00B1). Cells were sub-cultured and counted on a Beckerman-Coulter Z2 particle counter (range 10μm-30μm). Cells were plated at 10,000/cm² in a tissue cultured Greiner Bio-One T-75 flask and maintained in MSC-GRO™ serum free, complete medium (Vitro Biopharma Cat. No. SC00B3) in a reduced O_2 environment (1%O_2, 5%CO_2, 94%N_2) at 37°C in a humidified chamber. The MSCs were treated continuously with activation agents (including NutraVivo ™ Brain Grow Activator) for up to 2 weeks. Cultures were fed every three days. Cells were harvested using Accutase (Innovative Cell Technologies Inc., Cat No AT-104) for 15 minutes and placed into a 15mL conical tube. Cells were collected via centrifugation (450 x g) for 7 minutes. Cell supernatant was aspirated off and cells were resuspended in 1mL PBS and counted on a Beckerman- Coulter Z2 particle counter (range 10μm-30μm).

cDNA preparation and q-PCR analysis

Total RNA was extracted using RNeasy Mini Kit (Qiagen Cat. No. 74104). RNA was quantified using an absorbance measurement at 260nm. RNA was converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen Cat. No. 205310) in a thermocycler. cDNA was sent to an outside lab (CU-Anschutz Metabolic Laboratory) for q-PCR to detect relative or absolute gene expression levels. cDNA was diluted 1:5 and iTaq Universal Supermix fluorescent probe (BioRad Cat. No. 172-5120) was used to detect the threshold cycle (Ct) during PCR. Dilution factors and cDNA concentrations were calculated into recorded values then normalized to untreated hMSCs (Vitro Biopharma Cat. No. SC00A1). Values were imported into Origin 8.1 and graphed.

Effects of NutraVivo™ Dietary Supplementation on Stem Cells Isolated from Patients

Fat was donated by 3 female patients and processed for the extraction of AD-MSCs using collagenase digestion. One patient was on NutraVivo™ Dietary Supplements (NVF0525) for 6 months prior to fat collection. We compared the total cell counts, viability, total and viable mononuclear cells, doubling time, and isolated AD-MSCs post expansion between the NutraVivio™ patient and control patients. All AD-MSCs were analyzed through passage 2 (P2).

RESULTS

Cell Migration

Migration of MSCs exposed to Substance P is shown in the upper portion of Figure 1. Cells failed to migrate into the cell-free central zone of the well without substance P (Cell proliferation was blocked by mitomycin C) while 30 % closure occurred into the cell free zone when exposed to 50 nM Substance P. Dose-response curves following 24-hour exposure showed an EC50 of 5.8 to 5.9 pg/ml for the cell lines used (Figure 2). Since this is comparable to prior reported values using a different assay, the results of Figure 2 provide validation of the cell migration assay. Also, another similar study was performed that provides further validation of our cell migration assay. (Results not shown.)

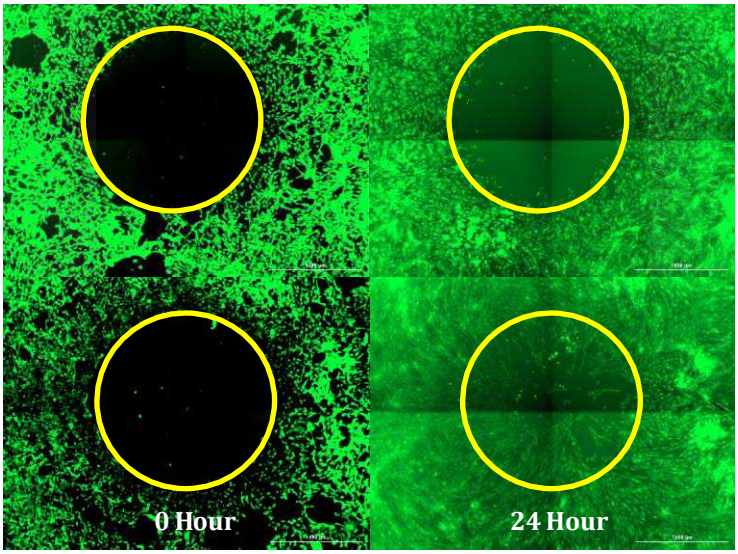

Figure 1: The images of cell migration using fluorescent readout and cell tracker green as a fluorescent marker of the MSCs.

These cell migration images show fluorescent human MSCs (green) at the beginning of the assay (left panel) of the Control vs. Activating Agent (Substance P at 50nM)) and 24 hours later (right panel). MSCs migrated to the cell-free center of the well and also filled open areas in other regions of the culture as a result of Substance P exposure but did not similarly migrate in its absence (upper right panel).

Figure 1 shows that cell migration extent is positively correlated with the dosage of activation agent. Increasing concentrations of Substance P resulted in dose-dependent percent closure, which is a quantitative measure of cell occupancy of the cell free zone in the center of the well.

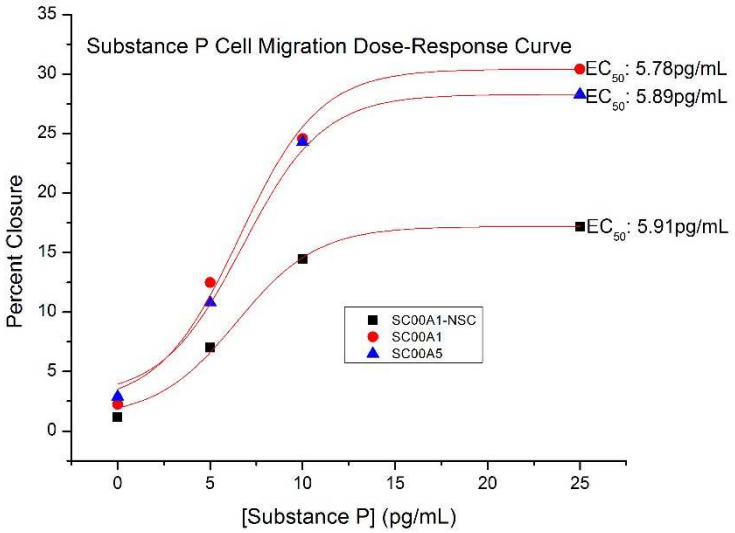

Figure 2: Migration of various human cell lines exposed to Substance P. The data shows the dose-response curve of U C-MSCs (Red circles), human primary pancreatic fibroblasts (blue triangles), and human NSCs (black triangles) together with EC_{50} values. Per cent closure was determined at 24 hours as described.

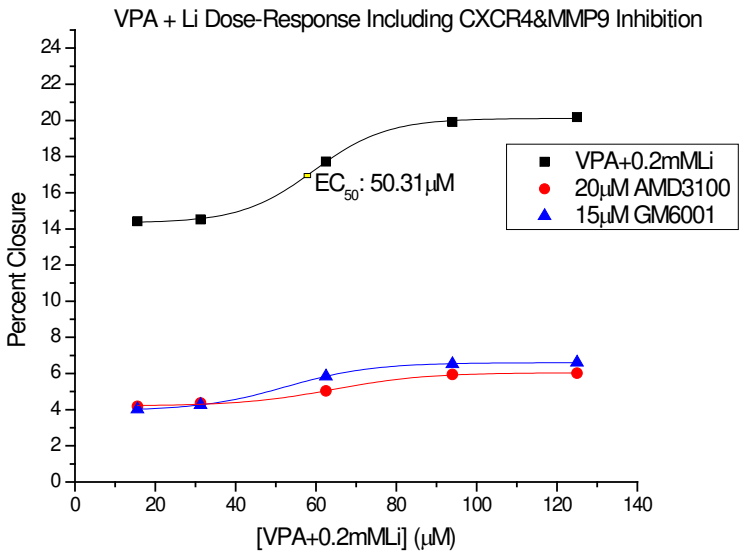

Figure 3: Graphic representation illustrating migration of UC-MSCs induced by exposure to VPA and 0.2mM lithium (black squares), VPA and 0.2mM lithium inhibited by CXCR4 inhibitor (AMD3100) (red circles) and VPA and 0.2mM lithium inhibited by MMP9 inhibitor (GM6001) (blue triangles). Percent closure is plotted as a function of dose and the data was modeled by sigmoidal curve fitting to calculate EC_{50} values. This Figure 3 shows VPA and lithium-induced MSC migration with a calculated EC_{50} of 50.31 μM and maximal migration in the combination of VPA and lithium.

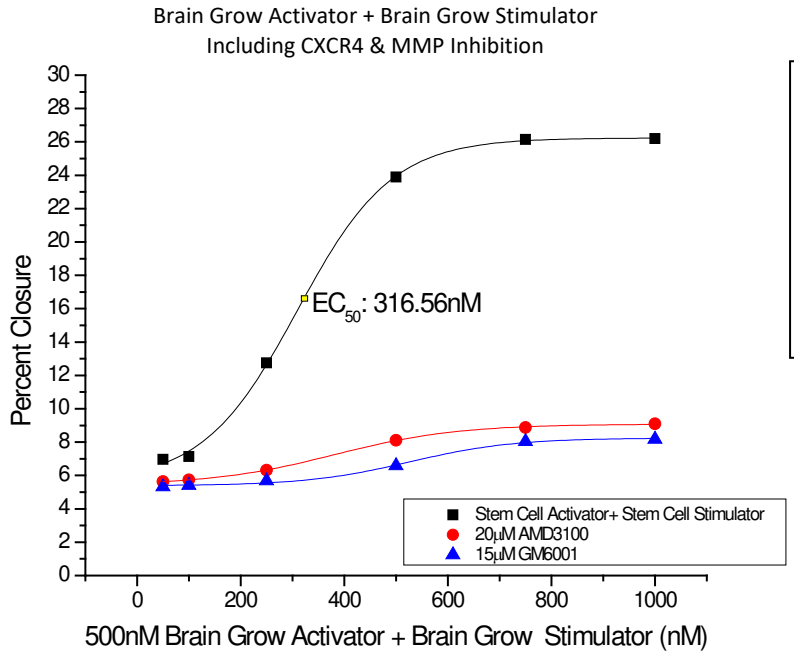

Figure 4: Migration of UC-MSCs induced by exposure to 500nM Stem Cell Activator and variable concentrations of Stem Cell stimulator (black curve) using sigmoidal curve fitting to calculate EC50 values. This yields a calculated EC50 of 316.56 nM. Maximal migration required both Brain Grow Activator and Brain Grow Stimulator. The CXCR4 inhibitor (AMD3100) blocked stem cell migration as did the MMP9 inhibitor (GM6001).

Proliferation

Using the same dose-response format from the cell migration assays, an EC_{50} was determined by relative fluorescent units (RFU) of Presto Blue staining, a fluorescent marker of cellular indication for quantitation of cellular proliferation. Day 0 measurements were subtracted from Day 3 measurements and the data was fitted to a sigmoidal curve to measure dose-response of target molecules.

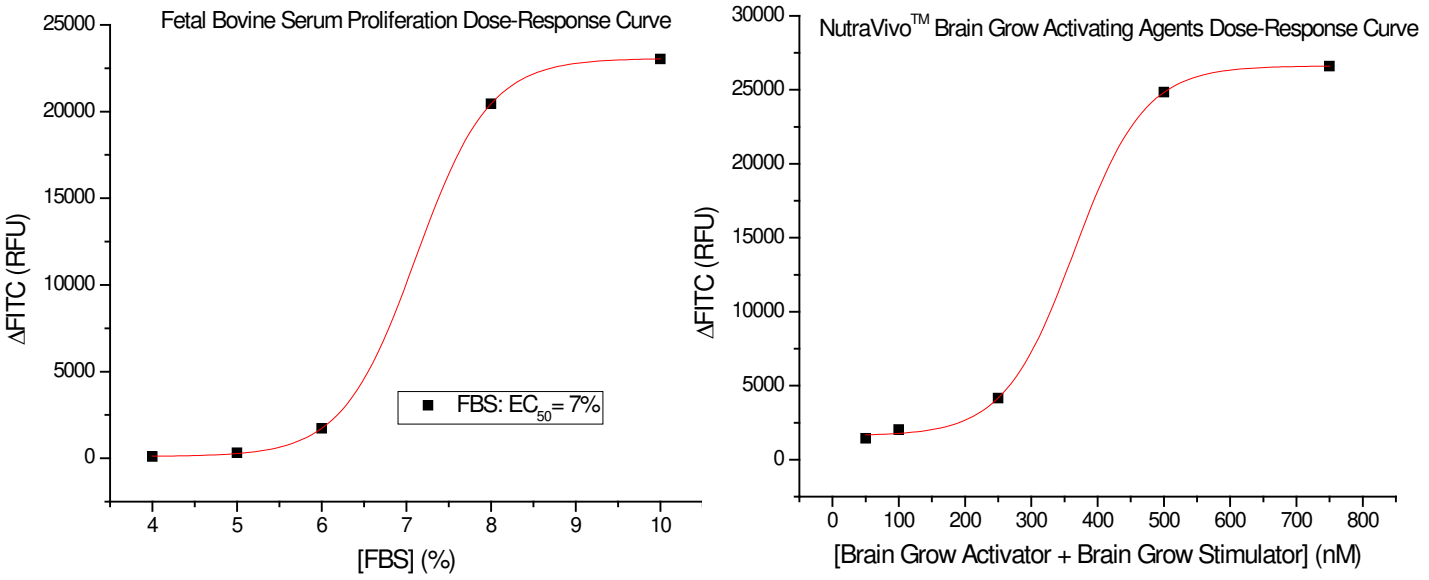

Figure 5 shows the proliferation effects of FBS (left) and Brain Grow Activator at 500 nM with variable concentrations Brain Grow Stimulator (right). Sigmoidal curve fitting was used to determine EC_{50} values. FBS ED_{50} was 7% while the ED_{50} for Brain Grow Stimulator together with 500 nM Brain Grow Activator was 370.3 nM and was comparable to the EC_{50} for cell migration, 316.6 nM.

Gene Expression Analysis

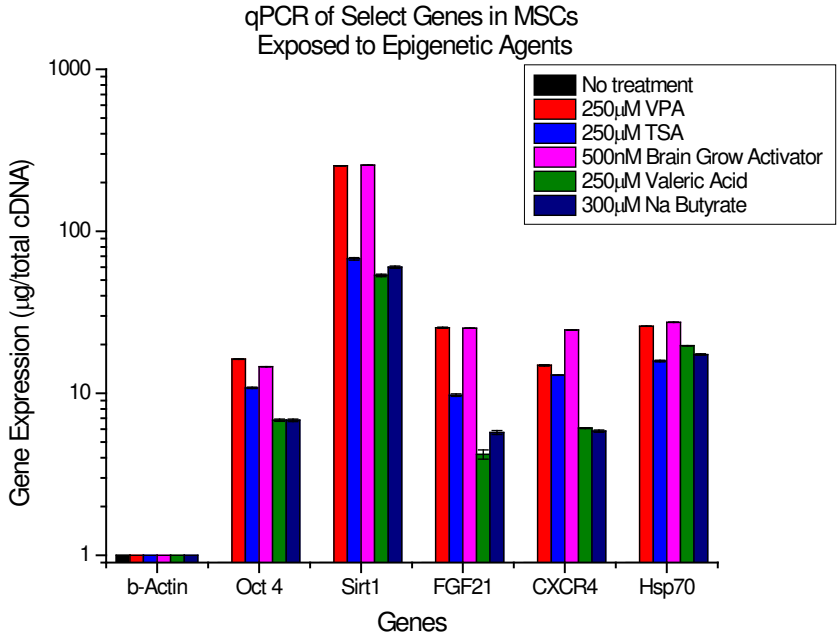

Figure 6: shows the expression levels of β-actin, Oct 3/4, Sirtuin I, FGF21, CXCR4 and Hsp70 in human MSCs exposed various epigenetic agents as determined by qPCR.

Figure 6 shows results of gene expression analysis following the exposure of MSCs to VPA and various nutraceuticals know to exhibit epigenetic effects. The expression of select target genes determined by qPCR is shown as compared to the house-keeping gene, β-actin. The expression of Oct 3/4, a well-known pluripotency gene, was increased about 20-fold compared to β-actin in human MSCs by VPA and NutraVivo™ Brain Grow Activator. TSA, valeric acid, and Na butyrate resulted in less expression activation. The expression of SIRT-1 was highly elevated by ~300-fold without differences between treatment with either VPA or Brain Grow Activator. While TSA, valeric acid, and Na butyrate were significantly less effective. Similar, yet lower expression levels of FGF-21, CXCR4, and Hsp70 were observed (maximum of 25-fold) with VPA and Brain Grow Activator showing nearly equivalent expression increases and lower levels with TSA, valeric acid, and Na butyrate. All gene expression was normalized to that of untreated MSCs. Beta-actin was used as a house-keeping gene. NutraVivo™ Brain Grow Activator showed equivalent or increased expression of Oct 3/4, SIRT-1, FGF21, CXCR4, and Hsp70 compared to VPA.

Fat Analysis of Patients Using NutraVivo™ Dietary Supplements

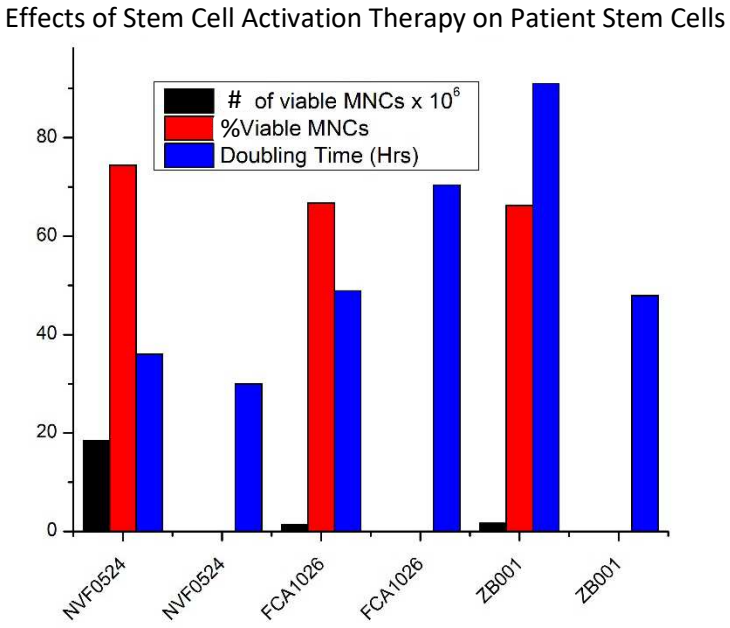

Figure 7 shows the effect of NutraVivo™ "Stem Cell Activation Therapy" on stem cells derived from patients. Patient with ID number NVF0524 was treated with NutraVivo™ Brain Grow Activator, Brain Grow Stimulator, and Brain Grow Energizer for 6 months while patient ID numbers FCA1026 & ZB001 did not have the NutraVivo™ stem cell activation therapy. The NutraVivo™ stem cell activation therapy increased the number of recovered viable MNCs by more than 10-fold at higher viability (red bars) and with lower doubling times (blue bars). These results show that NutraVivo™ stem cell activation therapy improves the quality of stem cells derived from fat of a patient.

DISCUSSION

The results presented here provide evidence that a natural substance formulation consisting of NutraVivo™ Brain Grow Activator, Brain Grow Stimulator, and Brain Grow Energizer result in human stem cell activation by increasing stem cell migration, proliferation, and epigenetic reprogramming. These effects were demonstrated by the results obtained *in-vitro* assays and by stem cell analysis of a patient treated with this formulation.

We first developed a cell migration assay based on live cell analysis of the movement of fluorescent cells into the cell-free zone of tissue culture plates created by blocked cell attachment at the center of wells. To assure accuracy of migration analysis by this system, we validated the assay using Substance P-induced migration of fibroblasts. This a well-known biological response to injury, where Substance P release from the injury site causes migration of cells by activation of cellular migration. We compared the EC_{50} values obtained in our migration assay with those obtained by a different assay method based on Boyden chambers that measure cell migration across a membrane. Since the EC_{50} values we found using human neural stem cells, MSCs, and primary fibroblasts were nearly the same (5.8 to 5.9 pg/ml; Figure 2), and equivalent to that determined in Boyden chambers using human fibroblasts (Kohler, CM, et al., Eur. J. Pharmacol. 1193; 249, 281), we demonstrated validation of our assay based on cell migration into cell-free zones.

The combination of VPA and Lithium has been shown to synergistically improve recovery from TBI & ALS in animal models (Yu, F, et.al., J. Neurosurg. 119: 766-773, 2013; Feng, H.L., et al., Neurosci 155: 567-572, 2008). We thus determined effects of VPA and Li on MSC migration where we also found evidence for synergistic effects on stem cell migration (Fig 3). The molecular mechanisms of the effect of lithium and VPA were then investigated on stem cell migration. To understand the effects of nutraceuticals as a replacement to pharmaceuticals, NutraVivo™ Dietary Supplements were investigated to compare lithium and VPA results. Since a prior report suggested that VPA up-regulated CXCR4, a critical chemokine receptor involved with cellular mobility, and that lithium up-regulated MMP-9 (Tsai, LK**,** et al., Stroke 42(10): 2932-2939, 2011), the effects of known inhibitors of CXCR4 and MMP-9 on the migration of CB-MSCs were determined and the results are shown in Figure 3 & 4. The migratory effect of VPA was blocked by the CXCR4 inhibitor AMD3100, while the MMP9 inhibitor, GM6001, blocked the migratory response to lithium. We believe that stem cell migration plays an important role in the recovery from TBI and therapy of ALS through promotion of stem cell movement to sites of injury and inflammation. Stem cells are dormant within the body and when activated, migrate to sites of injury & inflammation.

The results presented in Figure 4 show that the natural substances, Brain Grow Activator and Brain Grow Stimulator also induce stem cell migration through similar mechanisms. The migration caused by Brain Grow Activator was also blocked by the CXCR4 inhibitor (AMD3100), and migration caused by Brain Grow Stimulator was blocked by the MMP9 inhibitor (GM6001). These natural substances also activated proliferation of stem cells (Figure 5) and increased expression of specific genes in stem cells as well. Interestingly, one of the same molecules responsible for stem cell migration (CXCR4) was highly expressed by Brain Grow Activator. Molecular mechanisms for stem cell activation include both CXCR4 and MMP9.

Both CXCR4 and MMP9 are likely to be involved in stem cell migration. The chemokine receptor molecule, CXCR4 is crucial player in the stem cell migration through the CXCR4-SDF1α axis. Hence activation of CXCR4 will increase gene expression promoting stem cell migration to regions of higher SDF1α concentration in both short and long term exposures. CXCR4 is an effector molecule that drives the migration of stem cells. Also, MMP9 is a well- known protease that may promote stem cell migration by digestion of protein obstacles within tissues. Hence the activation mechanism underlying both pharmacological agents (Li & VPA) and the natural substances, Brain Grow Activator & Brain Grow Stimulator, share common features to increase stem cell migration. Once stem cells are mobilized from various tissue niches, they naturally migrate to regions of inflammation and cancer stem cells, etc. At the site of injury, various stem cell regenerative mechanisms are triggered including secretion of biologically active substances that protect cells, reverse apoptosis, oxidative stress, etc. Also, stem cells differentiate into other cell types and interact through intercellular channels (tunneling nanotubes) to exchange mitochondria and exosomes into those cells needing repair.

The results obtained from the *in-vitro*, cell-based assays are also supported by the results obtained from a patient treated with Brain Grow Activator, Brain Grow Stimulator, and Brain Grow Energizer. The latter provides general increases in cellular energy by promoting mitochondrial function. This patient was on the NutraVivo™ formulation for six months and then had their stem cells processed from fat derived through lipoaspiration. Analysis of these stem cells compared to untreated patients supports the results of cell-based assays. We saw a greater than 10-fold increase in the number of viable MNCs recovered from the lipoaspirate as well as a 2-fold increase in the rate of MSC proliferation (Figure 7). The former result is likely due to increased stem cell content within fat tissue due to increased stem cell proliferation, as demonstrated in the cell-based assays (Figure 4). Also, the increased MSC proliferation rate reflects increased stem cell potency.

CONCLUSIONS

The natural stem cell activators NutraVivo™ Brain Grow Activator, Brain Grow Stimulator, and Brain Grow Energizer, activate human adult stem cells by increasing proliferation and migration. These supplements yield increased numbers of mobilized stem cells with potential therapeutic benefits to various conditions including TBI, ALS, PD and other conditions characterized by inflammation. The mechanisms of activation involve increased expression and activity of CXCR4 and MMP9 with specific enhancements of stem cell migration to sites of injury and inflammation. The combination of NutraVivo™ Dietary Supplements improves cellular health through anti-oxidant and anti-aging effects including increased expression of the anti-aging gene Sirt I.

Abbreviations:

HSC, Hematopoietic stem cells: These reside in bone marrow and differentiate into blood cells only.

ESC, embryonic stem cells: Found within the embryo and they are pluripotent and immortal.

NSCs: neural stem cells: An adult stem cell residing in specific brain structures. They may differentiate into neurons, glial cells and endothelial cells.

G-CSF: Granulocyte, colony stimulating factor. A differentiating factor directing HSCs to white blood cells.

BMP11: Bone morphogenic protein-11 aka GDF11: Growth differentiating factor 11. This blood-borne growth factor cytokine activates Satellite cells, adult stem cells that differentiate into muscle cells.

GSK3-beta: Glycogen synthetase 3-beta: A critical regulator of the beta-catenin pathway that is essential to gene expression control.

HDAC-I: Histone deacetylase I: An enzyme that regulates DNA methylation and is important in the epigenetic regulation of gene expression.

Substance P: A neuropeptide with multiple effects as a neurotransmitter and potent vasodilator. It is also a chemokine attractant to migrating cells.

SDF1a: Stromal-derived factor 1 alpha: A chemokine with specificity to the chemokine receptor CXCR4 that in present in stem cells.

CXCR4: Chemokine receptor 4: A driver of cell migration towards regions of high chemokine concentration.

Oct ¾: A pluripotency gene that controls differentiation potential of stem cells.

Sirtuin I: An anti-aging gene. Its expression is increased by restricted caloric diets or resveratrol.

FBS: Fetal bovine serum.

VPA: valproic acid

Li: lithium

TSA: Trichostatin A

MMP9: Matrix metallopeptidae 9: a protease that dissolves extracellular matrices.