



## **CELL-BASED FUNCTIONAL ASSAYS OF STEM CELLS: Quantitative Analysis of Stem Cell Activating Agents**

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### **Abstract**

Bone marrow-derived stem cell therapies have been used for over 50 years in the treatment of blood disorders including leukemia, lymphoma and auto-immune disorders. Embryonic stem cells 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 applications 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 tend to form tumors following transplantation while adult stem cells have gained significant support for efficacy in the treatment of skeletal muscular disorders and several other indications. There are limitations using adult stem cell transplants because of cost, potential safety issues and the necessity of autologous transplants. Biological products activating endogenous hematopoietic stem cells 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 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. These assays have application in quality control testing of stem cells for cell therapy, quality control of stem cell activation agents, i.e., nutraceuticals, pharmaceuticals, and pathway-specific small molecule 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, epigenetic reprogramming and stem cell secretome analysis. Many *in-vivo* adult stem cells reside in quiescence with 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. And 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 chemokines and their receptors, e.g., the well-known interactions between SDF1alpha and CXCR4. Activated stem cells migrate to sites of inflammation and exert anti-inflammatory and other biological responses leading to 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 as a result of 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 is under active investigation. The epigenetic, proteomic and physiological interactions critical to 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. The need to better understand the process of stem cell activation has led to the development of live cell-based assays. These assays improve investigation methods with high throughput technology that allow the determination of precise doses of activating agents and other applications as well.

## **Materials and Methods**

### Cell Culture

Native human cord blood-derived MSCs (Vitro Biopharma Cat. No. SC00A1), human pancreatic fibroblasts (Vitro Biopharma, Cat. No. SC00A5), MSC-derived neural stem cells (Vitro Biopharma, Cat. No. SC00A1-NSC), colorectal cancer-associated fibroblasts (Vitro Biopharma, Cat. No. CAF05), and pancreatic stellate cancer-associated fibroblasts (Vitro Biopharma, Cat. No. CAF08) 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°C incubation for 15 minutes and placed into a 15mL conical tube. Cells were collected via centrifugation (450 x g) for 7 minute. 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

10<sup>6</sup> cells/cell line 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 (ThermoScientific, Cat. No.. 165305) containing cell seed stoppers (Platypus, Cat. No. CMAUFL4) to form a cell free zone and placed in 5%CO<sub>2</sub>, 1%O<sub>2</sub>, 94%N<sub>2</sub> at 37°C in a humidified chamber for 24hrs. Wells used for NSC cultures, were first treated with 10µg/mL Fibronectin (Sigma, Cat. No. F0556) for 2 hours at 37°C. Following washout with PBS (3x), cell seed stoppers were inserted, NSCs were plated at 25,000/well and incubated in 5%CO<sub>2</sub>, 1%O<sub>2</sub>, 94%N<sub>2</sub> at 37°C in a humidified chamber for 48hrs.

#### 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 purchase from Tocris Bioscience, (Cat. No. 1156) and curcumin from Santa Cruz Biotechnology- (Cat. No. SC-200509A). A TopSeal (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 and bright field data acquisition. The gas phase throughout the acquisition of kinetic data was 5% O<sub>2</sub>, 5% CO<sub>2</sub> with the balance nitrogen maintained by a BioTek CO<sub>2</sub>/O<sub>2</sub> gas controller. Images were saved as TIFF files and imported into the Image-J program 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/cell line 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<sub>2</sub>, 1%O<sub>2</sub>, 94%N<sub>2</sub> 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 activating agents and growth factors. Cells were stained with 10µL PrestoBlue/well (Invitrogen, Cat. No. A13261) and incubated in the dark in a glove box at 1%O<sub>2</sub>, 5%CO<sub>2</sub> 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, 3 and 5.

## **Reprogramming 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/cm<sup>2</sup> a tissue cultured Greiner Bio-One T75 flask and maintained in MSC-GRO serum free, complete medium (Vitro Biopharma Cat. No. SC00B3) in a reduced O<sub>2</sub> environment (1%O<sub>2</sub>, 5%CO<sub>2</sub>, 94%N<sub>2</sub>) at 37°C in a humidified chamber. The MSCs were treated continuously with activation agents 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 minute. 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) 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 graphed using Origin 8.1

## **Secretome Analysis**

### Cell Culture and Conditioned Media

Native human cord blood-derived MSCs (Vitro Biopharma Cat. No. SC00A1) were plated at 1,000/well in a tissue cultured 6-well plate (BD Falcon, Cat Number 353046) in MSC-Gro™ low serum, complete medium (Vitro Biopharma Cat. No. SC00B1). Cells were continuously grown for a period of 30 days. Condition media was collected at day 3, 6, 12, 18, and 24.

### MicroArray Analysis

Multiple microarrays were run using conditioned media for cytokine secretion determination after a continuous 24 day culture period. An inflammation microarray (Ray Biotech, Cat. No. QAH-INF-3), bone metabolism microarray (Ray Biotech, Cat. No. QAH-BMA-1) and a Th17 microarray (Cat. No. QH-TH17-1) were used to analyze the conditioned media. A laser scanner was used to measure the fluorescent signals of each microarray (Molecular Probes, Genepix 4000B).

## **Results and Discussion**

### **Cell Migration**

The EC<sub>50</sub> was determined for treatment with activating agents in the presence of the cell proliferation inhibitor, 5µg/mL mitomycin C. Kinetic data allowed determination of the optimal incubation time and closure for further experiments. An initial analysis of the cell free zone using Image J software was used to screen concentrations of activating agents for dose-response determination in further experiments. It was determined that hMSCs plated at 25,000/well, incubated for 24 hours and then exposed to appropriate concentrations gave optimal results. The same concentrations were used in a proliferating assay to determine a similar dose-response curve (shown below).

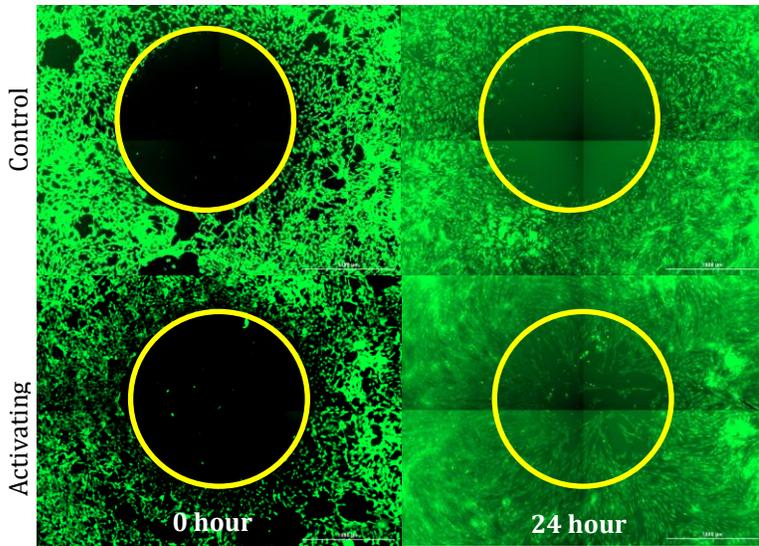


Figure 1: The images of cell migration using fluorescent readout and cell tracker green as a fluorescent marker of the MSCs. These cell migration images show fluorescent human MSCs (green) at the beginning of the assay (left panel) of the Control vs. Activating Agent (Substance P at 50nM) and 24 hours 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 2 shows that cell migration extent is positively correlated with the dosage of activation agent. Increasing concentrations of activation agent resulted in dose-dependent percent closure, which a quantitative measure of cell occupancy of the cell free zone in the center of the well.

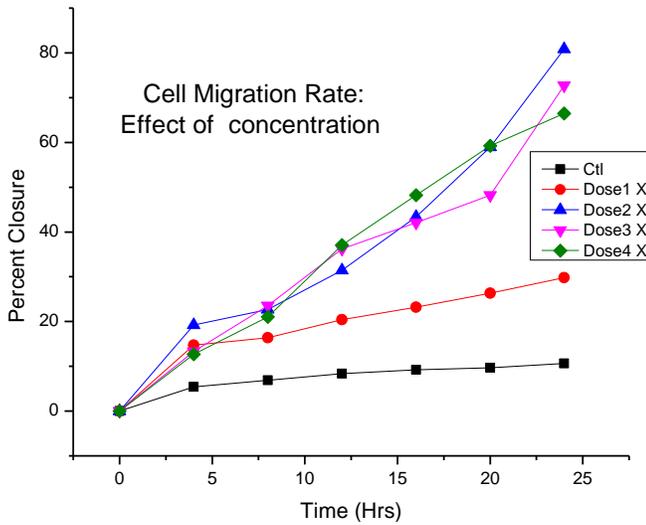


Figure 2: Image J analysis of activating agent's dose response. hMSCs were imaged in the 96-well microplate format using wide-field fluorescent microscopy and analyzed using Image J software. Images of wells subjected to 5µg/mL mitomycin C only were control wells in which little to no migration was exhibited. Percent closure was calculated for each concentration of activators, using Image J software and plotted as function of time. These data were then used to determine dose-response (shown below).

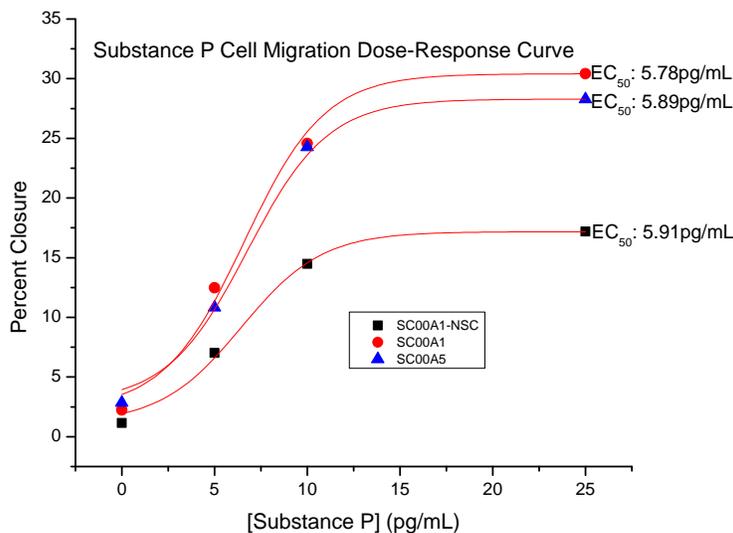
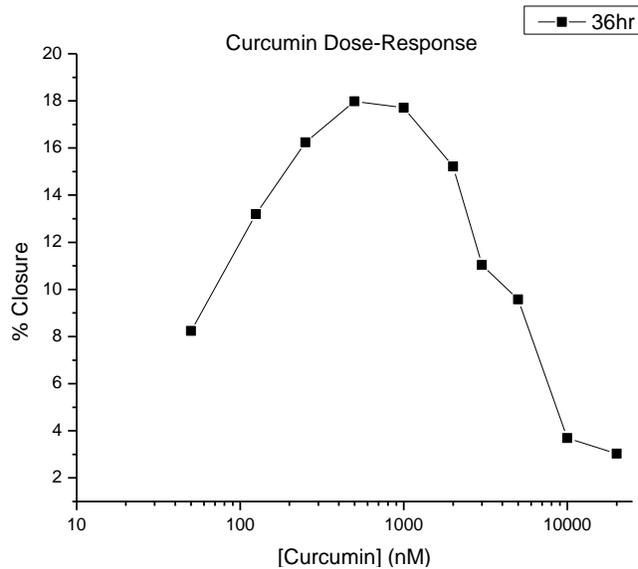


Figure 3: Migration of various human cell lines exposed to Substance P. The data shows the dose-response curve of CB-MSCs (Red circles), human primary pancreatic fibroblasts (blue triangles), and human NSCs (black triangles) together with EC<sub>50</sub> values. Percent closure was determined at 24 hours as described.



*Figure 4: Less than 1  $\mu\text{M}$  curcumin induced migration of human CB-MSCs and 1 to 10  $\mu\text{M}$  blocked migration due to apparent toxicity. Percent closure was determined after a 36 hour run period. An  $\text{EC}_{50}$  of 250nM for migration and an apparent  $\text{LD}_{50}$  of 3 $\mu\text{M}$  were calculated using Origin 8.1*

The results shown in Figures 3 and 4 provide validation results for our cell migration assay. The well-known stem cell migration inducing agent Substance P was used to determine  $\text{ED}_{50}$  for CB-MSCs, NSCs, a human primary pancreatic cell line and cancer-associated fibroblast (CAF) cells derived from a colorectal adenocarcinoma tumor. Substance P induced migration of all cells tested except CAFs. Migration of these cells was comparable to un-treated control cultures (data not shown). While there is growing evidence suggesting stem cell-like properties of CAFs, these results suggest an absence of migration comparable the stem cells and primary fibroblasts. The  $\text{EC}_{50}$  values for migrating cells were comparable and ranged from 5.78 to 5.91  $\mu\text{g/ml}$  ( $\sim 40$  nM). We also determined the effects of curcumin on MSC migration. Our results shown in Figure 4 indicate two effects of curcumin depending on its concentration. At low concentrations, it promotes stem cell migration while at higher concentrations,  $> 1 \mu\text{M}$ , it blocks migration through apparent toxicity. It's  $\text{ED}_{50}$  for migration was 250 nM and the apparent  $\text{LD}_{50}$  for toxicity was 3  $\mu\text{M}$ . These values compare favorably with literature reports on neural stem cell activation and toxicity by curcumin (Ormond, DR, et al., PLOSOne 9(2):e88916

**Proliferation**

Using the same dose-response format from the cell migration assays, an  $\text{EC}_{50}$  was determined by relative fluorescent units (RFU) on a Modulus Microplate Reader. Day 0 measurements were subtracted from day 5 and the data was fitted to a sigmoidal curve to represent dose-response of the growth factor FGF-basic (FGF-b).

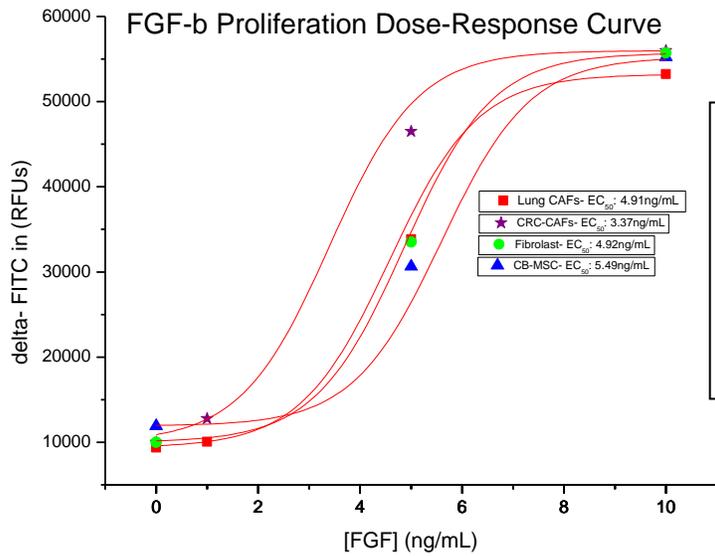


Figure 5: Dose-response curve of FGF-b for different cell lines after a 5 day period. Relative fluorescent units were measured using a FITC filter on a Modulus Microplate Reader. Origin 8.1 analysis plots concentration vs. delta FITC in order to achieve a dose-response curve EC<sub>50</sub> falls within 2 ng/mL of each other. Colorectal CAFs may be more sensitive to FGF-b than the other cell lines tested.

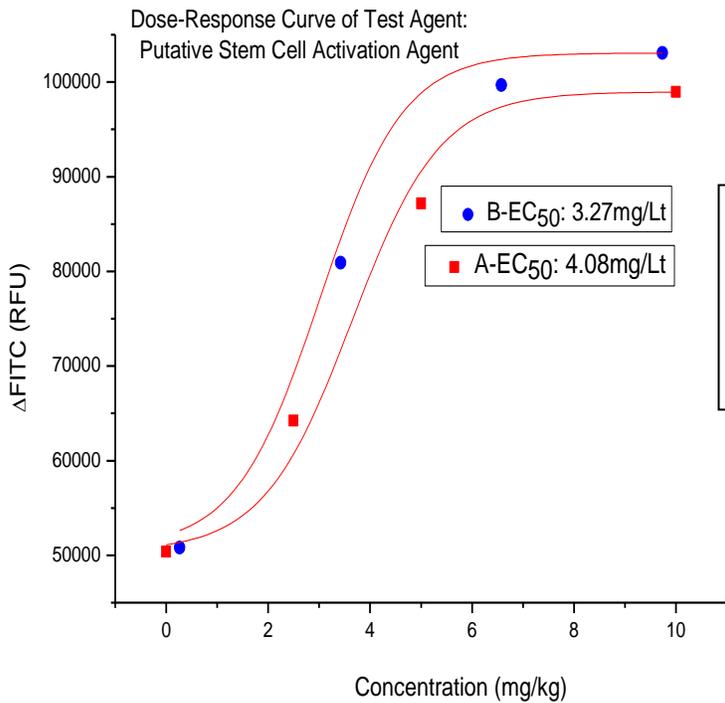


Figure 6: Dose-response curve of a putative activation agent on CB-hMSCs. The change in RFUs at 0 and 5 days following exposure is plotted as a function of concentration in back-to-back runs A (red) and B (blue).

FGF-b shows (Figure 5) anticipated growth effects in the various cell lines tested, including CB-MSC, lung CAFs, colorectal CAFs and a primary pancreatic fibroblast cell line. An example of testing a putative stem cell activation agent is shown in Figure 6. Here the EC<sub>50</sub> dosages in both proliferation and cell migration were comparable and allow inferences as to appropriate clinical trial dosage, based on pharmacokinetic and uptake/metabolism considerations.

### Reprogramming

Cells were treated with a combination of activating agents and one agent alone to see if reprogramming could occur. The result of gene expression analysis of human MSCs treated with a single activating agent or both activating agents shows a higher expression of pluripotent gene OCT3/4, the anti-aging gene SIRT1, and fibroblast family gene, FGF21 compared to normal hMSCs (shown below).

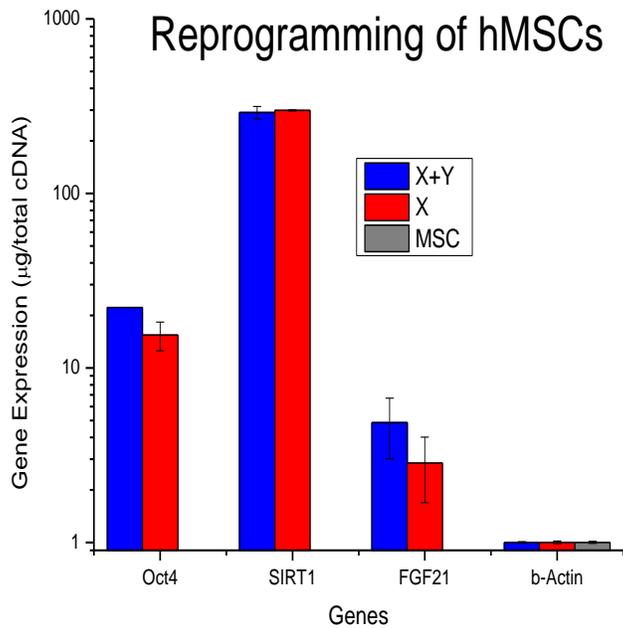


Figure 9: The graph shows the result of gene expression analysis of human MSCs treated with one activating agent vs combination of activating agents. The expression of Oct 3/4, a well-known pluripotency gene, was increased about 20-fold compared to untreated human MSCs. The expression of SIRT-1 was highly elevated compared to untreated MSC by ~300-fold without differences between one activating agent or both. The expression of FGF-21 was also elevated by 3 to 5-fold and its expression was higher in the combination treated cells, as previously reported, although this increase with not significant. All gene expression was normalized to untreated MSCs. These results thus show increased expression of Oct 3/4, SIRT-1 and FGF-21 as a result of exposure to individual activating agents or combination of activating agents.

### Secretome Analysis

Conditioned media was analyzed using an inflammation microarray, Th17 microarray and a bone metabolism array. Results show an increase in inflammatory cytokines as well as adhesion factors. With a significant increase in MIP-3a,

ICAM-1, IVCAM-1, and VE-Cadherin, this suggests the well-known immunosuppressive role of MSCs.

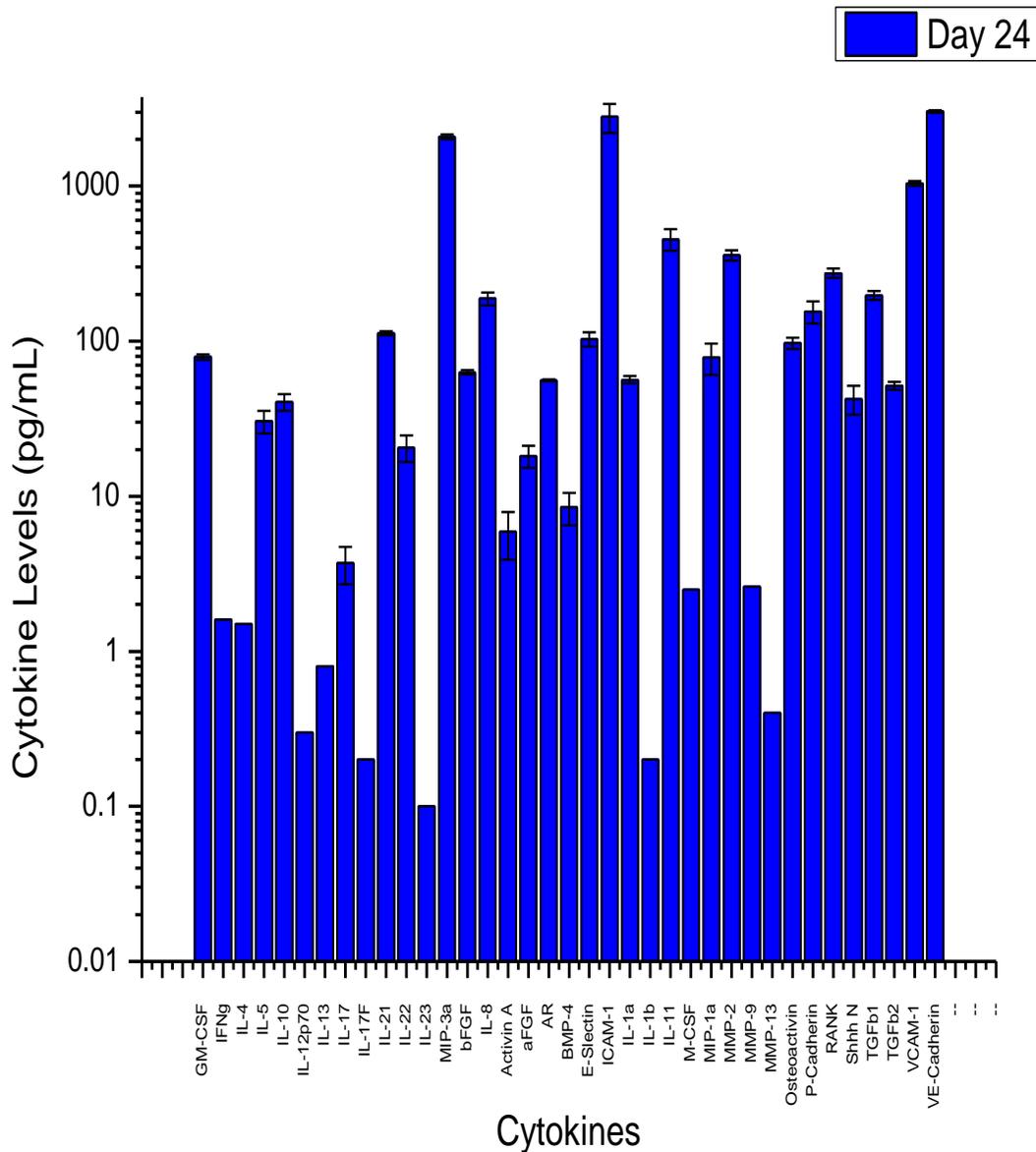


Figure 10: The graph shows cytokine secretion of hMSCs after a continuous 24 day culture. These results are the combination of all microarray analysis.

#### Conclusions:

1. Substance P induced migration of human MSCs, human pancreatic fibroblasts and MSC-derived human NSCs. However, a CAF derived from colorectal tumors failed to migrate within the Substance P concentration range tested. This may suggest some differences between CAFs and stem cells.
2. Curcumin induced stem cell migration at concentrations less than 1  $\mu$ M ( $ED_{50}$ = 250 nM) and blocked migration at higher concentrations with an apparent  $LD_{50}$  of 3  $\mu$ M.
3. FGF-b increased proliferation of lung and colorectal CAFs, fibroblasts and MSCs. The  $ED_{50}$  was ~5 ng/ml.
4. Some putative activation agents alter epigenetic control by inhibiting histone deacetylases resulting in altered gene expression. We found substantial acceleration of Oct 3/4 and Sirtuin I (SIRT1) expression and possible increased FGF-21 expression by a putative stem cell activation agent.
5. Cell culture media collected from activated stem cells showed increased secretion of cytokines involved with immunosuppression.

6. Combined assays of stem cell migration, proliferation and reprogramming together with biochemical analysis of secreted molecules offers a new window into stem cell functionality that can be utilized to developed advanced methods for activation of endogenous stem cells.

These cell-based assays provide suitable dynamics for activation of stem cells. Migration, proliferation, and reprogramming provide a platform for drug discovery and advancements in stem cell therapies. Using these assays could lead to the discovery of chemotherapeutic or activating agents.