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Human Mesenchymal Stem Cell Hydrogen Sulfide Production Critically Impacts the Release of Other Paracrine Mediators Following Injury

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Abstract

Background: Use of mesenchymal stem cells (MSCs) for treatment during ischemia is novel. Hydrogen sulfide (H_2S) is an important paracrine mediator that is released from MSCs to facilitate angiogenesis and vasodilation. Three enzymes, cystathionine-beta-synthase(CBS), cystathionine-gamma-lyase(CSE), and 3-mercaptopyruvate-sulphur-transferase(MPST), are mainly responsible for H_2S production. However, it is unclear how these enzymes impact production of other critical growth factors and chemokines. We hypothesized that the enzymes responsible for H_2S production in human MSCs would also critically regulate other growth factors and chemokines.

Materials and Methods: Human MSCs were transfected with CBS, MPST, CSE, or negative control siRNA. Knockdown of enzymes was confirmed by PCR. Cells were plated in 12 well plates at 100,000 cells/well and stimulated with TNF-α (50ng/ml), LPS (200ng/ml), or 5% hypoxia for 24 hours. Supernatants were collected and cytokines measured by multiplex beaded assay. Data were compared with Mann-Whitney and $p<0.05$ was significant.

Results: TNF-α, LPS, and hypoxia effectively stimulated MSCs. GCSF, EGF, FGF, GMCSF, VEGF, and IP-10 were all significantly elevated when CSE was knocked down during TNF-α stimulation ($p<0.05$). Knockdown of MPST during LPS stimulation more readily increased GCSF and EGF, but decreased GMCSF ($p<0.05$). CBS knockdown decreased production of GCSF, FGF, GMCSF, and VEGF ($p<0.05$) following hypoxia.

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Author Contributions:

TAM-Performed cell culture transfections, drafted manuscript, performed statistical analysis contributed to final version of manuscript

NAD-Performed cell culture transfections, performed PCR, contributed to final version of manuscript

ARJ-Isolated protein and assisted with Multiplex assay, contributed to final version of manuscript

KRO-Assisted with idea generation, reviewed data, contributed to final version of manuscript

DISCLOSURES

None

Conclusion: The enzymes that produce H_2S in MSCs are also responsible for the production of other stem cell paracrine mediators under stressful stimuli. Therefore, reprogramming MSCs to endogenously produce more H_2S as a therapeutic intervention could also critically impact other paracrine mediators which may alter the desired beneficial effects.

Keywords

hydrogen sulfide; stem cells; paracrine mediators; CBS; MPST; CSE

INTRODUCTION

The use of mesenchymal stem cells (MSCs) for the therapy of ischemic tissues has become a popular topic of study over the last decade (1–3). This interest comes from the lack of innovative drug therapies designed to treat these ischemic conditions. The benefit of stem cell therapy is that they release a multitude of paracrine mediators which function to limit cell death, promote tissue growth, and promote functional recovery of the injured organ (4). Our group has demonstrated a significant survival advantage when mesenchymal stem cells are used following intestinal ischemia (1). Furthermore, these advantages do not appear to be source tissue specific (5). The exact mechanism of stem cell mediated intestinal protection, however, has not been completely elucidated.

In this regard, one of the paracrine factors that MSCs are known to release is hydrogen sulfide (H_2S). H_2S has been found to be a novel therapeutic option for the treatment of intestinal injury as it can protect the intestines following both ischemia and necrotizing enterocolitis (NEC) in animal models (6, 7). Although hydrogen sulfide was originally thought to be a toxic gas, it has recently been found to be a beneficial gasotransmitter in the recovery of ischemic tissues. Cellular excretion is elevated in hypoxia, and it has been show to promote angiogenesis, cell migration, and vasodilation (8, 9). Hydrogen sulfide can be released from stem cells and also functions endogenously to maintain cell health (10–12).

Hydrogen sulfide is created enzymatically in humans via multiple conventional and unconventional pathways, of which three pathways are most prominent (13) (Figure 1). These pathways use different enzymes, namely cystathionine-beta-synthase (CBS), 3-mercaptopyruvate-sulphur-transferase (MPST), and cystathionine-gamma-lyase (CSE),to create hydrogen sulfide gas (14). Due to the extreme difficulties in measuring H_2S gas directly in biological systems, these three enzymes have been accepted as markers of cellular production of H_2S (14, 15). All utilize cysteine as a precursor to form H_2S gas. This gas can then be released from stem cells locally to provide beneficial effects during injury.

Although H_2S gas has clearly been shown to be a beneficial paracrine mediator, it is unclear how activation of the intracellular enzymes that make hydrogen sulfide affect the release of other stem cell growth factors and chemokines which are also beneficial for end organ protection. We therefore hypothesized that the enzymes responsible for H_2S production in MSCs would also critically regulate production of multiple other vital growth factors and chemokines following injury.

MATERIALS AND METHODS

Cell Culture

Human bone marrow-derived mesenchymal stromal cells (hBMSCs) were obtained from and authenticated by Dr. Darwin Prokop at Texas A&M University. Cells were cultured in polystyrene flasks in alpha-MEM (Life Technologies, Carlsbad, CA) with 16% FBS (Atlanta Biologicals, Flowery Branch, GA), 1% Glutamine and 1% pen/strep (Sigma, St. Louis, MO) at 37°C in 5% CO_2 in air. Once cells reached 90% confluence, they were lifted from their flask using TrypLE Express (Life Technologies, Carlsbad, CA). Cells were used between passages 2 and 10.

Cells were then pelleted at 400g for 5 mins and resuspended in fresh media. Cells were counted with the aid of an automated fluorescent cell counter (Luna-FL Automated Cell Counter, Logos Biosystems, South Korea), and 2.25 million live cells were resuspended in fresh media within a 225cm^2 polystyrene flask and allowed to adhere overnight.

siRNA Transfection and Cell Stimulation

After cells became adherent to the culture flask, they were prepared for small interfering ribonucleic acid (siRNA) transfection. siRNA to CBS, MPST, CSE, and a non-targeting control, (also known as Scramble) (GE Healthcare Dharmacon, Lafayette, CO), were resuspended in 1x siRNA buffer at a concentration of 20uM. siRNA was then combined with Dharmafect 1 (GE Healthcare Dharmacon, Lafayette, CO) in serum free media and allowed to stand at room temperature for 20 minutes. This was then added to normal medium and applied to the cells for a final concentration of 25nM. Transfection was carried out for 24 hours. The siRNA was then removed, cells were washed with PBS, and fresh media was applied. Cells were incubated for an additional 24 hours, and then were transferred to 12 well plates at a concentration of 100,000 cells/well. Cells became adherent to the culture plates and then were stimulated by tumor necrosis factor-α(TNF-α;50ng/ml), endotoxin(LPS;200ng/ml), or hypoxia (5%) for 24 hours as previously described (16). After this period, supernatants were collected into microcentrifuge tubes, centrifuged at 400g, and stored at −80C until analyzed.

Polymerase Chain Reaction and Assessment of Enzyme Knockdown

To confirm knockdown of CBS, MPST, and CSE, total RNA was isolated from cells using RNeasy Mini Kit with on-column DNase digestion (Qiagen, Hilden, Germany). Superscript III First Strand Synthesis kit (ThermoFisher Scientific, Waltham, MA) was used in order to reverse-transcribe 10 ng of each sample of RNA into cDNA using a random primer mix (Invitrogen, Carlsbad, CA). PCR was conducted using GoTaq Green MasterMix (Promega Corporation, Madison, WI) components and a hot-lid start on the LifeECO Thermal Cycler-Bioer Technology (Bulldog Biosciences, Porstmouth, NH). A positive control GAPDH primer set and primers specific for CBS, MPST, and CSE were used for subsequent PCR reactions (Integrated DNA Technologies, Coralville, IA, Table 1). An initial incubation of 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, Ta_{primer} for 45 seconds (annealing temperature specific to each primer), and 72°C for 1min was used with a final extension at

72°C for 5 min to finish the reactions. Reactions were run on a 2% agarose gel with Ethidium Bromide and visualized using Image Lab software 3.0 (Bio-Rad, Hercules, CA).

Multiplex Beaded Assay

Cells were lysed and homogenized in RIPA buffer (Sigma, St. Louis, MO) with protease and phosphatase cocktail inhibitors (1:100 dilution, Sigma). Homogenates were centrifuged at 12,000 rpm to pellet cell debris, and supernatants were transferred to fresh microcentrifuge tubes for storage at −80C. Total protein concentration was then quantified by Bradford Assay using a spectrophotometer (Versamax microplate reader, Molecular Devices, San Jose, CA). Soluble granulocyte colony stimulating factor (GCSF), epidermal growth factor (EGF), fibroblast growth factor (FGF), granulocyte/monocyte colony stimulating factor (GMCSF), vascular endothelial growth factor (VEGF), and interferon gamma induceable protein 10 (IP-10) were quantified with a Bioplex 200 multiplex beaded assay system (Bio-Rad, Hercules, CA) using multiplex plates for inflammatory chemokines and growth factors (Millipore, Burlington, MDA). This assay system is a well-established tool that uses colored beads that are coated with specific capture antibodies (17). The results can be read by flow cytometry because the beads are distinguishable by fluorescent signature. Assays were performed at 1:25 dilution according to the manufacturer's instructions and repeated to insure accuracy (N=6 per group).

Statistical Analysis

All statistical analysis was done using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Cytokine data reported as the mean +/− SEM and compared using the Mann-Whitney U test for nonparametric variables. P-values less than or equal to 0.05 were considered statistically significant.

RESULTS

Transfection of hBMSCs knocked hydrogen sulfide-producing enzymes

Transfection of hBMSCs with siRNAs for CBS, MPST, and CSE effectively decreased mRNA expression of these enzymes in individual transfection groups (Figure 2). The transfection process itself though did not result in significant differences in any measured cytokine level between non-stimulated control groups and Scramble transfection groups (Figure 3).

TNF-α Stimulation

Stimulation of hBMSCs with TNF-α significantly increased GCSF, GMCSF, and IP-10 above control values ($p<0.05$, Figure 3). Other cytokines assessed were not altered with TNF-α stimulation. In the presence of TNF-α, the transfection process itself decreased production of GMCSF compared to Scramble.

Knockdown of CBS did not alter GMCSF, EGF, GMCSF, or FGF compared to Scramble following TNF-α stimulation. However, VEGF was significantly decreased, and IP-10 was significantly increased compared to Scramble ($p<0.05$). Knockdown of MPST significantly increased GCSF, FGF, VEGF, and IP-10 production compared to Scramble ($p<0.05$) but had

no effect on EGF and GMCSF. Knockdown of CSE predominantly affected cytokines after TNF-α stimulation. All measured cytokines were significantly increased following CSE knockdown (Figure 4).

LPS Stimulation

LPS stimulation decreased a number of cytokines, including FGF, GMCSF, and VEGF compared to control (Figure 3). Conversely, LPS did increase production of IP-10 ($p<0.05$). In the presence of LPS, the transfection process significantly increased GCSF, FGF, GMCSF, VEGF, and IP-10 in cells transfected with Scramble siRNA compared to non-transfected controls (Figure 3).

Knockdown of CBS had only minor effects on cells after stimulation with LPS. GCSF was significantly decreased compared to scramble after LPS stimulation, while IP-10 was significantly elevated. CBS knockdown had no effects on other measured cytokines following LPS stimulation. Knockdown of MPST significantly increased GCSF and EGF, significantly decreased GMCSF, and had no effect on VEGF, IP-10, and FGF following LPS stimulation. Conversely, CSE ablation resulted in increased FGF and VEGF production from hBMSCs, and no change in GCSF, EGF, GMCSF, and IP-10 (Figure 5).

Hypoxic Stimulation

Hypoxic stimulation was found to decrease FGF and GMCSF levels ($p<0.05$). In the presence of hypoxia, the transfection process significantly increased all measured growth factors and chemokines compared to non-transfected controls (Figure 3).

When CBS was knocked down in the presence of hypoxia, GCSF, FGF, GMCSF, and VEGF levels all decreased ($p<0.05$, Figure 6). There was no difference in EGF or IP-10 levels in CBS knockdowns after hypoxic stimulation. Knockdown of MPST only resulted in a significant decrease in GMCSF compared to scramble, but didn't have a significant impact on other measured chemokines. Likewise, knockdown of CSE had no effect on chemokines compared to scramble.

DISCUSSION

Hydrogen sulfide has received more attention over the last several years for its beneficial biological properties. Typically thought to be a toxic gas that smells like rotten eggs, H_2S has been identified as the third gasotransmitter, in addition to the more popularly studied carbon monoxide and nitric oxide (14). Humans endogenously make hydrogen sulfide via multiple pathways (13). Three of these pathways have been more rigorously studied, yet it is still unclear how these pathways affect the production of other chemokines and growth factors. Herein, we observed that the enzymes that make H_2S are also actively involved in modulating the expression of other beneficial growth factors and chemokines.

TNF-α, LPS, and hypoxia are three stimuli that are often present in tissues during injury. Therefore, if stem cells are used therapeutically, these stimuli could likely serve to activate them *in vivo*. As expected, each stimulus induced the production of slightly different growth factors and chemokines from the stem cells. These stimuli work through various intracellular

cascades, such as NFκB, to induce the expression of unique profiles of paracrine mediators which aid in injury recovery (16).

VEGF, EGF, and FGF are three growth factors that are essential for organ recovery. VEGF promotes angiogenesis by inducing endothelial cell proliferation and migration, as well as sprouting of vascular tube formation (18). In the murine intestine, VEGF is highly expressed in the myenteric plexus, which may guide the ingression of vessels into the lamina propria during villous development. This phenomenon may maintain normal structure of the villous microvasculature during intestinal restitution (19). EGF has also been shown to be a major trophic factor for the intestine. Global EGF receptor deficiency in mice results in early neonatal death and a hemorrhagic enteritis that resembles human NEC (20). Furthermore, EGF administration following intestinal ischemia in a rat model demonstrated decreased inflammation as well as decreased intestinal destruction following injury (21). Other studies have shown similar results with a heparin bound form of EGF in models of necrotizing enterocolitis (22). Finally, fibroblast growth factor has been shown to increase intestinal epithelial cell restitution up to 10-fold (23). Enhanced expression of various FGF family members has been seen in the intestines of patients with inflammatory bowel diseases, suggesting a possible endogenous role for this family of factors after injury (23). In our study, genetic ablation of MPST and CSE significantly increased the expression of these growth factors, especially with TNF-α and LPS stimulation. This suggests that these enzymes, as opposed to CBS, are more prominent in limiting the expression of vital growth factors from stem cells during therapy.

Knockdown of CBS, MPST, and CSE also impacted the production of several stem cell chemokines. These included GCSF, GMCSF, and IP-10. GCSF initiates the proliferation and differentiation of leukocytes into mature neutrophils. It also induces hematopoietic stem cell mobilization from the bone marrow into the blood stream (24). Unlike GCSF, GMCSF also affects more cell types, including macrophages and eosinophils (25). Finally, IP-10, also known as CXCL10, is a known proinflammatory and antiangiogenic chemokine. It serves as a link between inflammation and angiogenesis (26). IP-10 inhibits vascular tube formation and VEGF-induced endothelial motility (27). In this regard, CSE plays a critical role in stem cell mediated GCSF, GMCSF, and IP-10 production when cells are stimulated with TNF-α. However CBS and MPST may play a bigger role in the production of these chemokines with LPS and hypoxic stimulation.

In this study, the transfection process itself was seen to effect changes in certain cytokines. This is not surprising, as transfection processes have universally been shown to be toxic to cells (28). A balance must therefore be achieved to provide cells with enough transfection reagent to have high transfection efficiency with low cytotoxicity. In this study we utilized a scrambled sequence as a transfection control in order to control for the transfection process. In the future, electroporation or viral delivery methods could be considered as alternatives, however, both have notable drawbacks (29).

Stem cells may work to protect injured tissues through the release of hydrogen sulfide. Genetic manipulation of the endogenous H_2S producing enzymes in mesenchymal stem cells clearly impacts the production of other growth factors and chemokines. Understanding how

genetic manipulation of these stem cells can impact other stem cell paracrine factors is of utmost importance for cellular therapy.

LIMITATIONS

Several limitations to this study exist, with the most notable being its *in vitro* nature. However, we felt that the assessment of these cells in culture would allow for the isolation of individual stimulation cascades. *In vivo*, cells are stimulated by a multitude of factors simultaneously, and it would be difficult to identify individual signaling pathways. An additional limitation is the study of only six chemokines and growth factors. Obviously there are a multitude of paracrine factors that could have been studied, but we felt that these key factors are likely the most important "snapshot" in terms of recovery following intestinal injury.

Finally, as noted, hydrogen sulfide is produced by multiple pathways. The three enzyme pathways studied herein are the most widely studied for H_2S production, and therefore were included in this study. A vigorous study of all hydrogen sulfide enzyme pathways would be out of the scope of this work.

CONCLUSIONS

Hydrogen sulfide is a beneficial gasotransmitter with important biological properties. The enzymes that are responsible for making hydrogen sulfide endogenously are also involved in the production and release of other chemokines and growth factors. Therefore, mechanisms that attempt to alter stem cell release of hydrogen sulfide will likely alter other beneficial pathways. These effects need to be carefully considered before genetic manipulation of stem cells is undertaken to overproduce hydrogen sulfide for therapeutic purposes.

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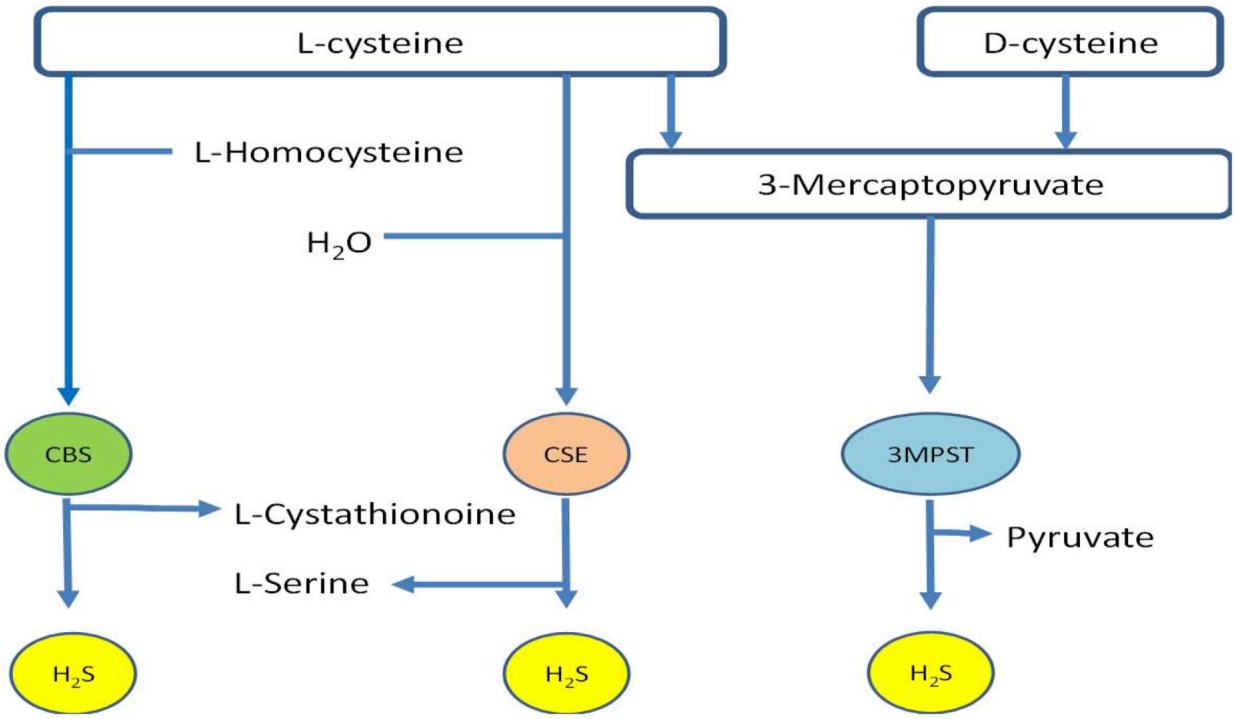

Figure 1:

Hydrogen sulfide is endogenously made via three enzymatic pathways. These pathways use different enzymes, namely cystathionine-beta-synthase (CBS), cystathionine-gamma-lyase (CSE), and 3-mercaptopyruvate-sulphur-transferase (MPST), to create hydrogen sulfide gas. All three of these enzymes utilize cysteine as a precursor.

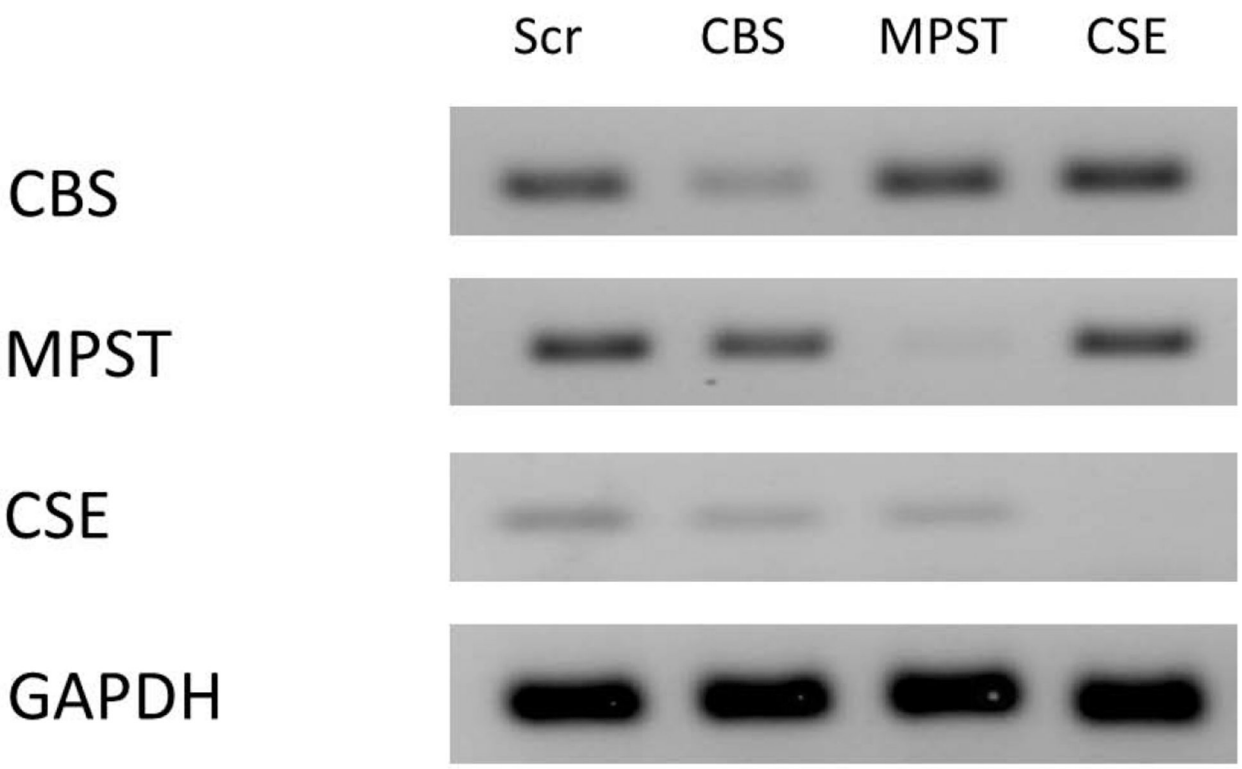

Figure 2:
Utilization of siRNA effectively reduced CBS, MPST, and CSE mRNA expression in hBMSCs compared to negative control (scramble-Scr). GAPDH was used as the housekeeping gene.

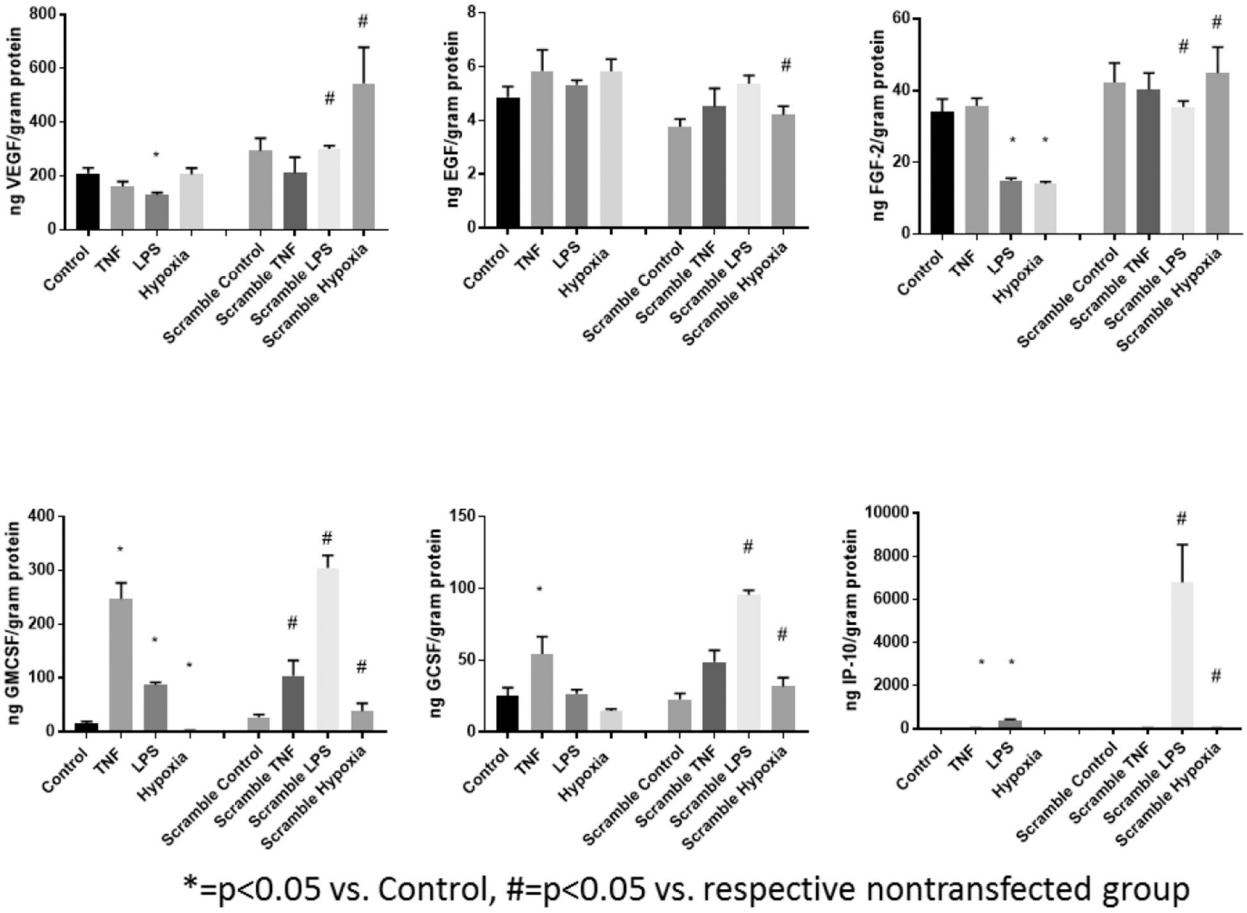

Figure 3:

Stimulation with TNF-α, LPS and hypoxia alters hBMSC cytokine expression. Transfection with negative control (Scramble) siRNA in the presence of these stimuli also impacts stem cell cytokine profiles. Mann Whitney was used for statistical analysis (N=6, *=p<0.05 vs. Control, #=p<0.05 versus respective non-transfected group)

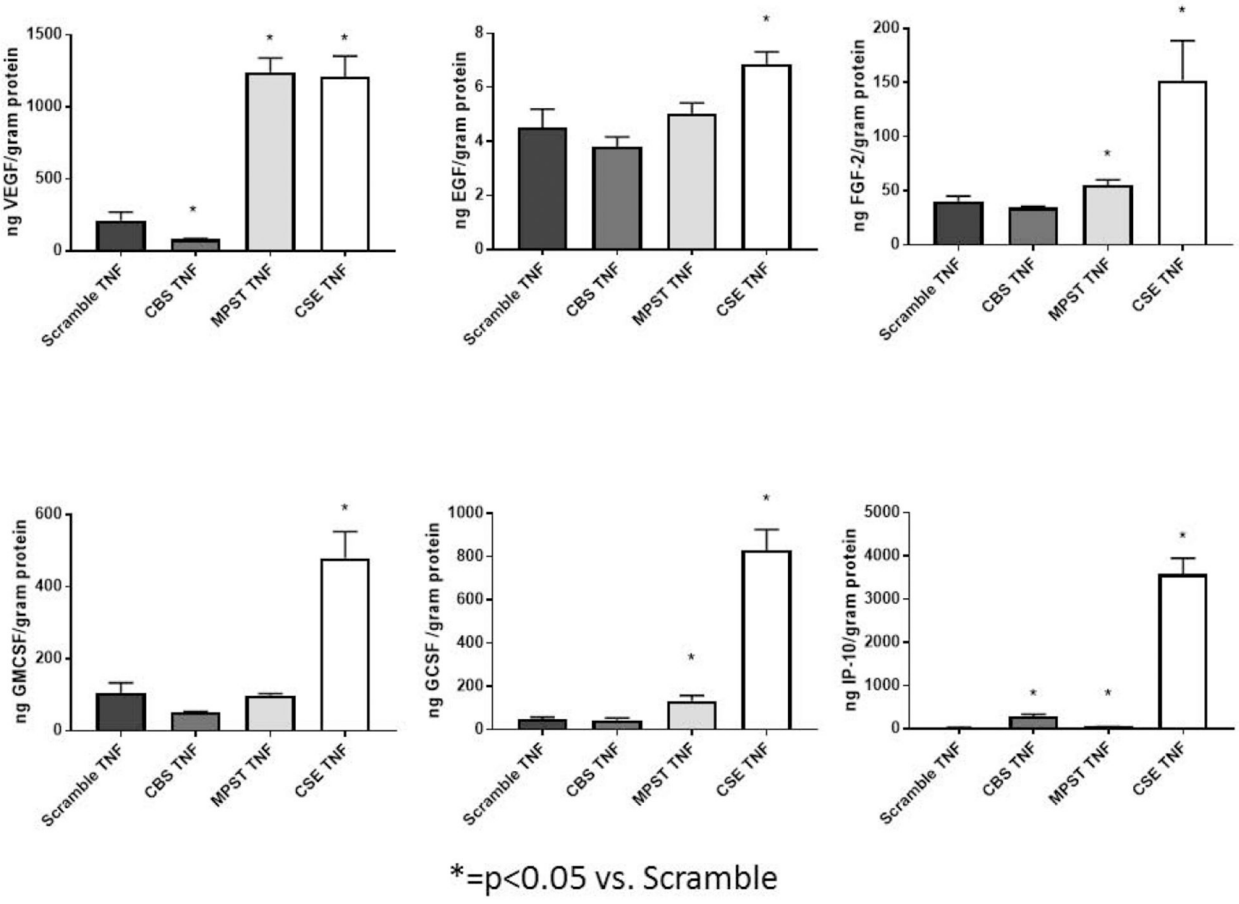

Figure 4:

Genetic Knockdown of CBS impacts hBMSC growth factor and cytokine expression. Mann Whitney was used for statistical analysis (N=6, *=p<0.05 vs. Scramble)

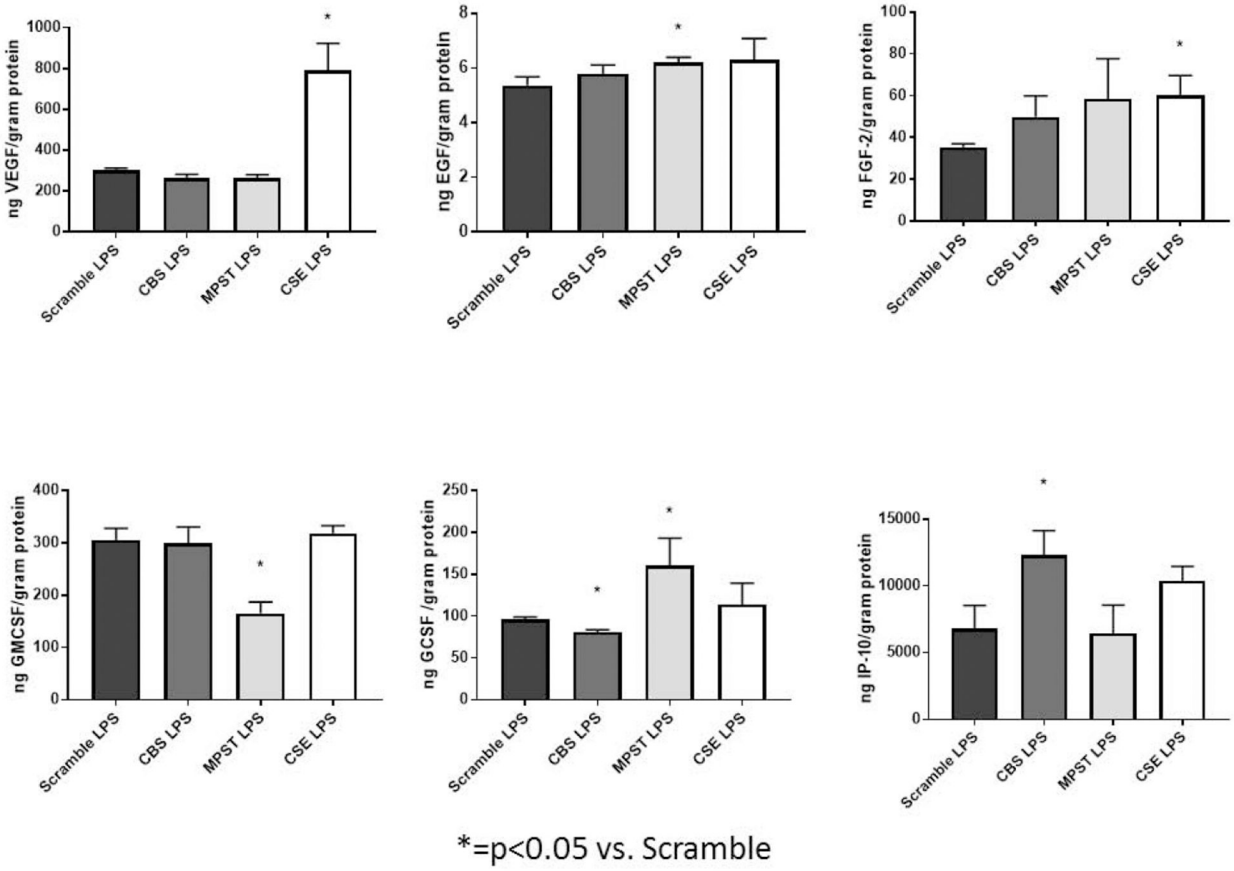

Figure 5:

Genetic Knockdown of MPST impacts hBMSC growth factor and cytokine expression. Mann Whitney was used for statistical analysis (N=6, *=p<0.05 vs. Scramble)

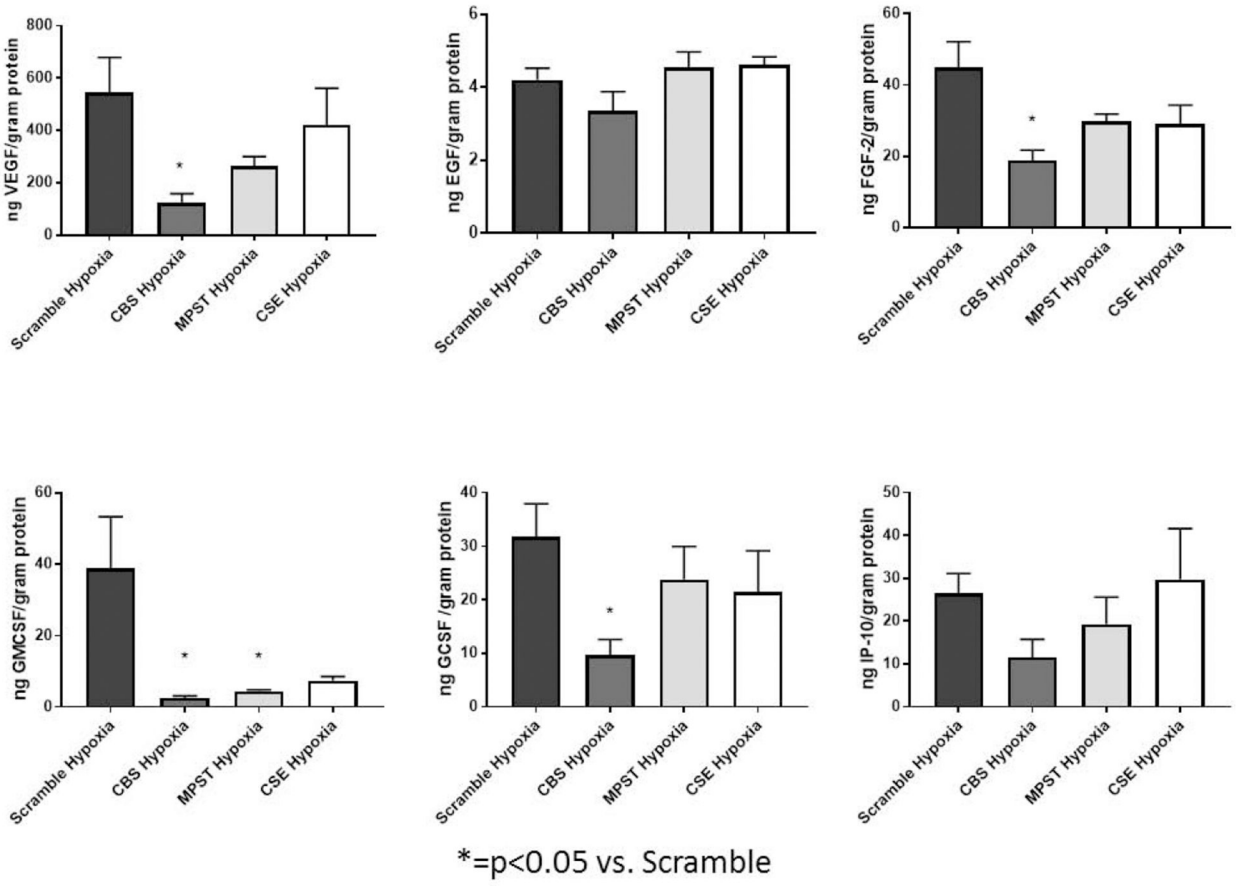

Figure 6:

Genetic Knockdown of CSE impacts hBMSC growth factor and cytokine expression. Mann Whitney was used for statistical analysis (N=6, *=p<0.05 vs. Scramble)

Table 1

CBS Primer Ta_{vrimer}: 50.7°C
CBS Forward: **5′- GTC AGA CCA AGT TGG CAA AGT −3′**
CBS Reverse: **5′- CAC CCC GAA CAC CAT CTG C −3′**
PrimerBank ID 295821199c3
MPST Primer Ta_{vrimer}: 51.4°C
MPST Forward: **5′- CGC CGT GTC ACT GCT TGA T −3′**
MPST Reverse: **5′- CAG GTT CAA TGC CGT CTC G −3′**
PrimerBank ID 372622377c1
CSE Primer Ta_{primer}: 51.2°C
CSE Forward: **5′- CAT GAG TTG GTG AAG CGT CAG −3′**
CSE Reverse: **5′- AGC TCT CGG CCA GAG TAA ATA −3′**
PrimerBank ID 299473757c3
GAPDH Primer Ta_{primer}: 50.7°C
GAPDH Forward: **5′- GGA GCG AGA TCC CTC CAA AAT −3′**
GAPDH Reverse: **5′- GGC TGT TGT CAT ACT TCT CAT GG −3′**
PrimerBank ID 378404907c1