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Slow and sustained nitric oxide releasing compounds inhibit multipotent vascular stem cell proliferation and differentiation without causing cell death

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ABSTRACT

Atherosclerosis is the leading cause of cerebral and myocardial infarction. It is believed that neointimal growth common in the later stages of atherosclerosis is a result of vascular smooth muscle cell (SMC) de-differentiation in response to endothelial injury. However, the claims of the SMC de-differentiation theory have not been substantiated by monitoring the fate of mature SMCs in response to such injuries. A recent study suggests that atherosclerosis is a consequence of multipotent vascular stem cell (MVSC) differentiation. Nitric oxide (NO) is a well-known mediator against atherosclerosis, in part because of its inhibitory effect on SMC proliferation. Using three different NO-donors, we have investigated the effects of NO on MVSC proliferation. Results indicate that NO inhibits MVSC proliferation in a concentration dependent manner. A slow and sustained delivery of NO proved to inhibit proliferation without causing cell death. On the other hand, larger, single-burst NO concentrations, inhibits proliferation, with concurrent significant cell death. Furthermore, our results indicate that endogenously produced NO inhibits MVSC differentiation to mesenchymal-like stem cells (MSCs) and subsequently to SMC as well.
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1. Introduction

Atherosclerosis, characterized by narrowing and hardening of the arterial wall, inflammation, and arterial plaque formation [1–3], is present in 90% of patients suffering from cardiovascular related illnesses. Current understanding states that the migration and proliferation of vascular smooth muscle cells (SMC) are key events in the formation and progression of atherosclerotic lesions [4]. For decades, scientific investigations have operated under the hypothesis that mature SMCs de-differentiate into a proliferative phenotype (pSMC) following denudation of the vascular endothelium. pSMCs migrate from the tunica media to the tunica intima, proliferate, and secrete extracellular matrix (ECM) proteins, which serve as the core and preliminary foundation of atherosclerotic plaques [5,6]. However, a recent study has introduced new evidence in support of an alternate theory, which suggests that atherosclerosis is caused by elevated stem cell differentiation [7].

The vascular tunica media was believed to be comprised of only mature SMCs. However Tang et al. [7] identified two medial non-SMC cell types, MVSC and MSC. Lineage-tracing experiments reveal several contradictions to the SMC de-differentiation theory. First, MVSCs can differentiate into MSCs and subsequently to mature SMCs. Second, MVSCs and pSMCs cannot be derived from the de-differentiation of mature SMCs. Third, MVSCs convert from a quiescent to an activated (proliferative and differential) state in response to endothelial denudation or *in vitro* isolation, and contribute toward vascular remodeling and neointimal formation. Finally, MVSCs and MSCs are capable of differentiating into other cell types including chondrocytes and osteoblasts, which would explain the arterial hardening that occurs in patients suffering from atherosclerosis. Taken together these findings introduce a new paradigm: MVSC differentiation, rather than SMC de-differentiation

Abbreviations: SMCs, smooth muscle cells; NO, nitric oxide; iNOS, inducible nitric oxide synthase; MTT, (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide); cDMSO, cellular grade dimethyl sulfoxide; LDH, lactate dehydrogenase; MSC, mesenchymal-like stem cell; MVSC, multipotent vascular stem cell; DETA-NO, diethylenetriamine NONOate; ABH, amino-2-borono-6-hexanoic acid; LPS, lipopolysaccharide; IFN-γ, interferon gamma; bFGF, basic fibroblast growth factor.

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contributes to vascular disease, and establishes a population of pSMCs [7,8]. Staunch proponents of SMC de-differentiation theory maintain its validity [9], and call into questions the methods and findings of Tang et al. [7]. Therefore, additional studies are needed.

The salutary role of nitric oxide (NO) as an inhibitor of oxidative stress [10], inflammation [11], proliferation [12] and platelet aggregation [13], has made it an ideal mediator against atherosclerosis [14]. Specifically, NO has been implicated in the inhibition of SMC proliferation [15,16]. We have recently reported the preparation of a variety of slow and sustained, low molecular weight NO releasing compounds [17–19], which have effectively inhibited SMC proliferation *in vitro* [18,19]. We report herein the inhibitory effect of NO on MVSC proliferation using three different NO-donors: the commercially available, diethylenetriamine NONOate (DETA-NO, **3′**), along with **1′** and **2′**, two slow and sustained NO donors prepared in our laboratory [18,19]. In addition, our report includes the inhibitory effects of endogenous NO on MVSC differentiation to MSC.

2. Materials and methods

2.1. Chemicals and equipment

Hyclone Dulbecco's Modified Eagle Medium (DMEM, Fisher), Fetal Bovine Serum (FBS, ScienCell), chick embryo extract (CEE, MP Biomedical), N2 (Invitrogen), B27 (Invitrogen), basic fibroblast growth factor (bFGF, Sino Biological Inc.), retinoic acid (Sigma–Aldrich), 2-mercaptoethanol (Sigma–Aldrich), Penicillin/Streptomycin (P/S, ScienCell) were procured. PureLink® RNA Mini Kit, TRIzol®, On-column PureLink® DNase Treatment, and High-Capacity cDNA Reverse Transcription Kit were purchased from Invitrogen. Primers were obtained from Integrated DNA Technology. MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay kit and cellular dimethyl sulfoxide (cDMSO) were purchased from ATCC. Lactate dehydrogenase (LDH) cytotoxic assay kit was supplied by BIOO Scientific. Diethylenetriamine NONOate (DETA-NO) and amino-2-borono-hexanoic acid (ABH) were purchased from Cayman Chemical. Lipopolysaccharide (LPS) and interferon-γ (IFN-γ) were obtained from Sino Biomedical Laboratories and Peprotech, respectively. Absorbance was determined using and Molecular Devices micro plate reader and SoftMax Pro software.

2.2. Isolation and culture of multipotent vascular stem cells

The target cells were obtained utilizing a tissue explant method outlined previously [20]. Briefly, rat aortae (kindly supplied by Professor Gary Dunbar) were dissected in a sterile environment. The tunica media was isolated from the accompanying connective tissue and endothelium, sectioned into 2 mm cubes, and placed onto a Cellstart treated 6 well plate, luminal surface down. Once attached, 1 mL of enhanced medium (EM, DMEM with 10% FBS and 1% P/S) was added to each well and plates were incubated. EM was replaced every four days. On day 15, EM was replaced with an equal volume of maintenance medium (MM) (DMEM with 1% FBS, 2% chick embryo extract, 1% N2, 2% B27, 20 ng/mL bFGF, 100 nM retinoic acid, 50 nM 2-mercaptoethanol, and 1% P/S) to prevent MVSC differentiation. Every 2–4 days any detached tissue was removed, and half of the medium was replaced. After 2–3 weeks any remaining explants were removed, and the entire volume of MM was replaced in each well. Cells were incubated until a suitable monolayer formed, at which point they were transferred to a CellBIND treated flask at a density of 8×10^5 cells/mL for culture expansion. Following passage six, cells were transferred to CellBIND treated 96 well plates (2500 cells/well), and incubated for 16–24 h to ensure attachment. The cellular population was synchronized with serum-free MM (100 μL), and incubated at 37 °C for 24–48 h prior to subsequent tests.

To allow for MVSC differentiation to MSC, MVSCs were allowed to grow in EM and harvested after six days. Therefore, MVSCs had a total exposure to EM for 21 days to yield the MSC control group. To activate inducible nitric oxide synthase (iNOS), MVSCs (in EM) on day 15 were treated with LPS (50 μg/mL) and IFN-γ (20 ng/mL) for 6 h. The medium was removed and ABH, a potent arginase inhibitor [18,21] (5 mM) dissolved in EM, was added and the cells were incubated for an additional six days.

2.3. Characterization of isolated vascular cells (qPCR experimental procedure)

Total RNA was isolated using PureLink® RNA Mini Kit, quantified using a Nano-drop ND-1000 spectrophotometer, and reverse transcribed to cDNA utilizing the High capacity cDNA Reverse transcription kit (Applied Biosystems) and random primers (Supl. Mat.). All qPCR reactions were conducted in triplicate using Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific). Thermo cycling was performed on an ABI 7500 Real-time PCR System (Applied Biosystems) starting at a hold stage of 95 °C for 10 min, and 40 cycles of denaturation at 95 °C for 15 s, annealing/extension at 60 °C for 60 s followed by disassociation melt curve analysis. Negative controls (no template) were also conducted in triplicate for all target genes.

The C_t for each sample was determined by taking the mean of three technical repeats. The data was normalized to 18S ribosomal RNA, and relative expression was assessed against MSC cells in EM at 3 weeks using the $2^{-\Delta\Delta Ct}$ method [22]. Data are the averages of two biological replicates, repeated three times. Data were analyzed using a Student's *T* test where $p < 0.05$ was considered significant.

2.4. MVSC exposure to secondary amines

Solutions of secondary amines **1** and **2** were prepared in cDMSO at three concentrations (10, 20, and 40 μM and 0.5, 1.0, and 2.0 mM, respectively). DETA (**3**) was dissolved in cellular grade water (cH_2O) to prepare 0.5, 1.0, and 2.0 mM solutions. An aliquot of each amine solution was combined with MM to render a 1% solution (v/v). The serum-free medium was replaced with 100 μL of each sample medium solution and plates were incubated for 48 h, followed by the addition of 100 μL of fresh sample containing medium to each well for a final volume of 200 μL. Plates were again incubated for 48 h, before conducting MTT or LDH analysis for cell viability or cell death assay respectively, according to the manufacturer's instructions and as described elsewhere [18].

2.5. MVSC exposure to NO-donors

Solutions of NO-donors (*N*-nitrosated amines), **1′**, **2′**, and **3′** were prepared at the same concentrations, and added to synchronize MVSCs in the same manner as their corresponding amines.

3. Results and discussion

3.1. Characterization of proliferation and differentiation of isolated cells

In order to authenticate that the isolated vascular cells were MVSCs, which can proliferate and differentiate to MSCs, we took recourse to quantifying the expression of seven genes. Cells treated in MM from day 15 (MVSC) and those treated in EM for 21 days

(MSC) were tested for the expression of MVSC markers, Sry-related HMG box 10 and 17 (Sox10 and Sox17 respectively), neural filament-medium polypeptide (NEFM), calcium binding protein B (S100β), calponin-1 (CNN1) and smooth muscle α-actin (SMA) were used to monitor differentiation, while Ki67 acted as a proliferation marker. Results from these studies are displayed in Fig. 1. An examination of this figure indicates the following. The differences in expression of Sox10 and Sox17 were not statistically significant when comparing MVSCs and MSCs, while significantly higher expressions of NEFM, S100β, and Ki67 were observed in MVSCs. Furthermore, the high expression of Ki67 is indicative of the proliferative nature of isolated MVSCs. Consistent with earlier findings [7], CNN1 and SMA were expressed at significantly lower levels in MVSCs than in MSCs. However, our observations with Sox17 expressions are not consistent with previously reported significantly lower levels of Sox17 in MSC compared to MVSC [7]. It is highly likely that this sole inconsistency is due to the duration of exposure of MVSC to EM. In our study the medium for MVSCs was switched from EM to MM after 15 days, while Tang et al. [7] assessed MVSCs after switching the medium (from EM to MM) on day 5. It is possible that perhaps the expression of Sox17 decreases in MVSCs kept in EM for a longer duration.

3.2. MVSC exposure to NO-donors

Three different NO-donors were used in this study. After NO is released, each NO-donor converts to the corresponding secondary amine (Scheme 1), therefore it was essential to determine the effects of each amine (**1**, **2**, **3**) on MVSC proliferation. None of the amines exhibited discernible differences in cellular viability (Supp. data). Thus, any reduction in MVSC viability following exposure to one of the three NO-donors could be attributed solely to the NO released from each compound.

The results from MVSC exposure to each NO-donor are displayed in Fig. 2(A–C). Analysis of this figure clearly indicates that all three NO-donors decrease MVSC viability in a concentration dependent manner. However, loss of cell viability can be due to a combination of proliferation inhibition and cell death. Therefore, to accurately determine the extent of MVSC proliferation inhibition, it was necessary to determine how much of the overall reduction in MVSC viability was due to cell death. Toward this end we took recourse to LDH cytotoxicity assays. Data from these studies are displayed in Fig. 2D. The decrease in viability (results of MTT assay), overall cytotoxicity (LDH assay), and the calculated proliferation inhibition (all expressed as percentages) are shown in Fig. 2E. An examination of the data indicates that the percent cytotoxicity values are statistically equal to that of 1% DMSO (negative control) at the two lowest concentrations for **1**′, and all three concentrations for **2**′, both of which are slow and sustained NO releasing compounds. On the other hand, the extent of cytotoxicity for compound **3**′ was greater than that of its negative control (1% cH_2O) and increases in a concentration dependent manner reaching 4.2% cell death at the highest concentration (20 μM). The observed cytotoxicity must be therefore be ascribed to the large single-burst NO release from the commercially available **3**′. In contrast, previously reported studies have found **3**′ to cause SMC cell death (50%) at 30 μM [23]. The markedly different cytotoxic behavior of **3**′ clearly demonstrates the difference between our isolated MVSC and commercially available SMC.

3.3. Effects of endogenous nitric oxide on MVSC proliferation and differentiation

In order to generate endogenous NO in MVSC, iNOS was induced by treating MVSCs with LPS and IFN-γ. ABH was used to block arginase activities to ensure a higher available L-arginine pool for NO production [21]. Subsequently, ABH exposed MVSCs were subjected to MTT assays to assess (ABH) concentration dependent anti-proliferative effects of endogenous NO derived from iNOS catalyzed conversion of the arginine pool. A reduction of MVSC cell viability (~24%) could be achieved when ABH concentration reached 5 mM (Fig. 3). In addition, MVSCs exposed to the same set of conditions, were analyzed by qPCR to determine the effects of endogenous NO on MVSC proliferation and differentiation to MSCs (Fig. 1). An examination of this figure indicates ABH treated cells expressed significantly lower levels of proliferation marker Ki67, compared to MVSCs not treated with ABH (control). Furthermore, Sox17 has been shown to increase reconstitution and renewal potentials in mouse multipotent adult hematopoietic stem cells [24] and when overexpressed causes increased tumor

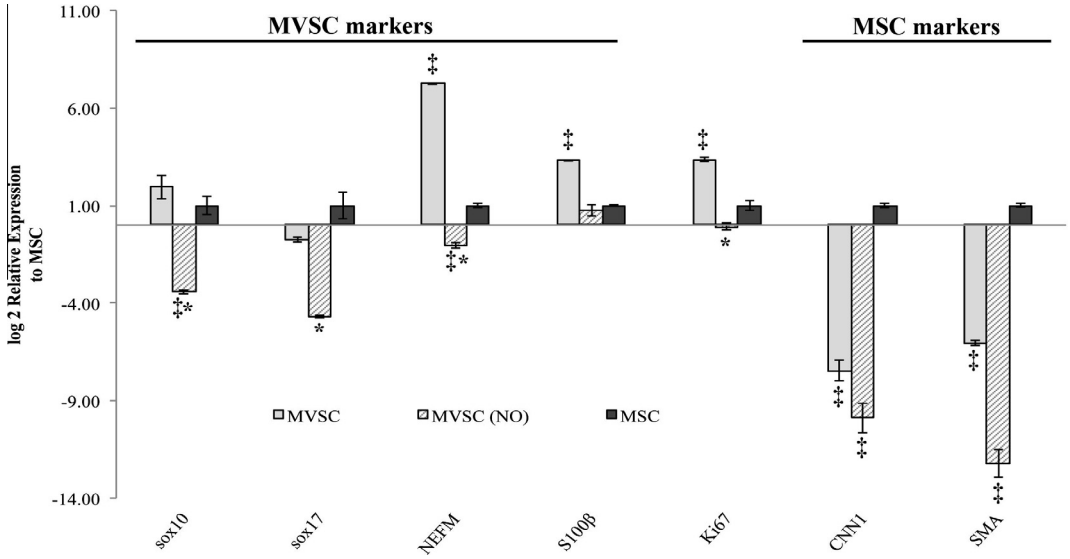
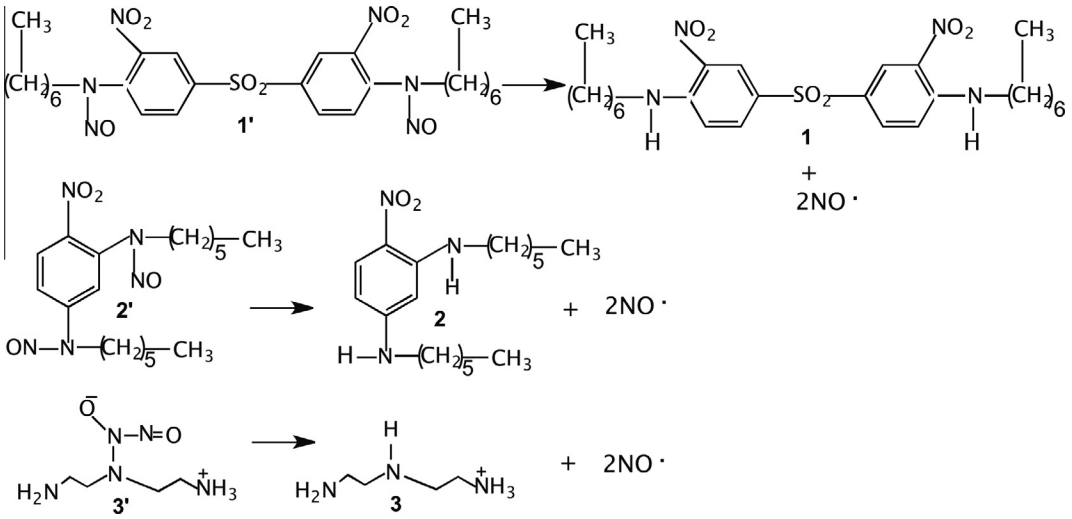
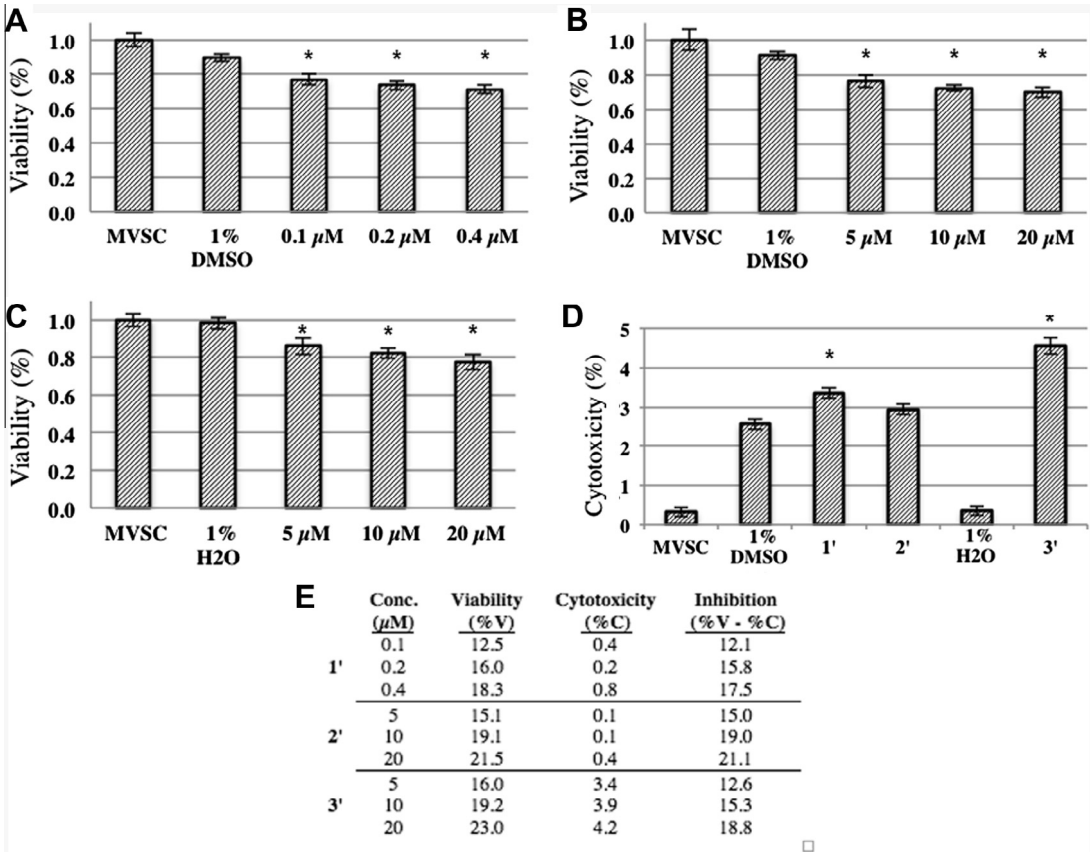


Fig. 1. Quantitative RT-PCR expression of MVSC markers and MSC differentiation markers in MVSC, MSC and endogenous NO treated MVSCs (MVSC (NO)], at 21 days. Gene expression was normalized to 18S ribosomal RNA and is the log 2 relative expression compared to differentiate MSCs, grown in EM, depicted graphically as expression at 1. Data are mean of two cell cultures, experimentally replicated three times, ±SD. '‡' indicates a significant difference set at $p < 0.05$ in expression from MSC, assessed by a Student's *T* test. '∗' Indicates a significant difference set at $p < 0.05$ from MVSC, assessed by a Student's *T* test.



Scheme 1. Release of NO from NO-donors **1′**, **2′**, and **3′**.



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	Conc. (μM)	Viability (%V)	Cytotoxicity (%C)	Inhibition (%V - %C)
1'	0.1	12.5	0.4	12.1
	0.2	16.0	0.2	15.8
	0.4	18.3	0.8	17.5
2'	5	15.1	0.1	15.0
	10	19.1	0.1	19.0
	20	21.5	0.4	21.1
3'	5	16.0	3.4	12.6
	10	19.2	3.9	15.3
	20	23.0	4.2	18.8

Fig. 2. Concentration dependent anti-proliferative effects of NO-donors on cultures of MVSCs, using MTT assay, cDMSO (1% in MM) or cH_2O (1% in MM) were used as the negative control. Changes in MVSC proliferation were evaluated after a 96 h incubation period with a given NO-donor (in cDMSO). Absorbance measurements of each well (570 nm) provided data points, which were expressed as a percentage of the positive control (100%). (∗) Indicates a statistically significant difference ($p < 0.05$) between the given compound/concentration combination and the vehicle alone. The graphical data represents the mean ± SD of triplicate determinations on the extent of MVSC proliferation inhibition in the presence of (A) **1′**, (B) **2′** and (C) **3′**, (D) cytotoxic effects of **1′**, **2′**, and **3′** (highest concentrations used) on cultures of MVSC using LDH assay, (E) summary of percent inhibition of proliferation by the NO-donors and their corresponding percent cytotoxic effects on MVSC.

growth and angiogenesis in mouse tumor vessels [25] – indicative of proliferation. Therefore, the significantly reduced expression of Sox17 in ABH treated cells compared to MVSC control also points toward these cells being in a non-proliferative state. These finding in conjunction with the MTT assay data above, further confirms the inhibitory effects of both endogenous NO (ABH) and exogenous NO (NO-donors) on MVSC proliferation.

Experimental evidence has demonstrated the effects of NO on stem cell activity [26,27]; the exact role of NO in determining the fate of these cells differs greatly between stem cell types. For

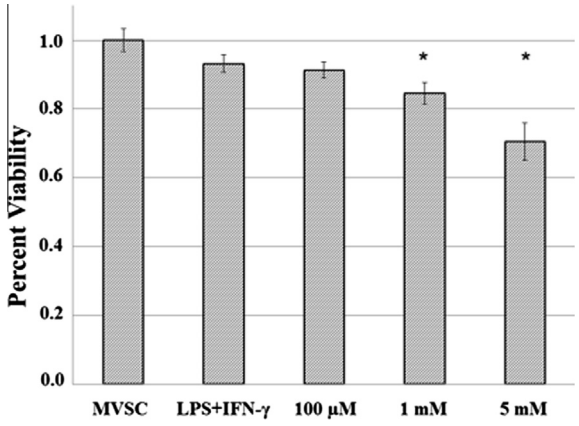

Fig. 3. Determination of anti-proliferative on cultures of MVSCs by ABH at varying concentrations utilizing MTT viability assay. (∗) Indicates a statistically significant difference ($p < 0.05$) between the given compound/concentration combination and the positive control. The graphical data represents the mean ± SD of duplicate determinations.

example, NO acts as an inhibitor of hematopoietic stem cell proliferation and differentiation in the bone marrow [28,29]. However, in embryonic stem cells NO has been shown to induce differentiation to cardiomyocytes [30]. In the case of nitroglycerin derived NO, human mesenchymal stem cell proliferation and osteoblastic differentiation are both stimulated through the nitric oxide pathway [31].

In the present instance, the ABH treated MVSCs, like those comprising the control group, showed a significantly decreased expression of MSC marker genes CNN1 and SMA compared to the MSC control (Fig. 1). The decreased levels of these MSC marker genes suggest that NO exposure inhibits MVSC differentiation to MSCs. To the best of our knowledge, this is perhaps the first report on the inhibitory role of NO on MVSC differentiation.

Treatment of MVSCs with ABH also rendered significant decreases in Sox10 and Ki67 expression in comparison to the control group of MVSCs. Additionally ABH treatment significantly decreased expression of NEFM (a MVSC marker), and showed a concurrent non-significant decrease of S100β expression. Taken together, these results suggest that the presence of NO not only prevents MVSC differentiation to MSCs, but also returns activated (proliferative and differential) MVSCs to the quiescent state displayed before endothelial denudation and/or *in vitro* isolation [7].

In summary, our studies confirm the earlier findings [7] that in response to endothelial injury or *in vitro* culturing, MVSCs are activated to a proliferative state and become capable of differentiating to MSCs, which can further differentiate to SMCs [7]. More importantly, NO inhibits both MVSC proliferation and their differentiation to MSC, as well as returning activated MVSCs to their homeostatic, quiescent state. These observations strengthen the new theory that atherosclerosis is caused by stem cell differentiation rather than SMC de-differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.087.

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