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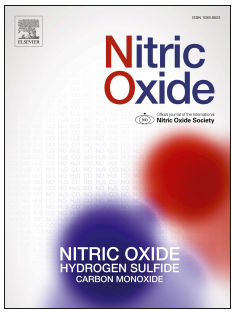

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Endothelial nitric oxide synthase induces heat shock protein HSPA6 (HSP70B') in human arterial smooth muscle cells

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Abstract

Endothelial nitric oxide synthase (eNOS) is the major source of nitric oxide (NO) production in blood vessels. One of the pleitropic functions of eNOS derived NO is to inhibit vascular smooth muscle cell proliferation in the blood vessel wall, and whose dysfunction is a primary cause of atherosclerosis and restenosis. In this study there was an interest in examining the gene profile of eNOS adenoviral (Ad-eNOS) transduced human coronary artery smooth muscle cells (HCASMC) to further understand the eNOS inhibitory effect on smooth muscle cell proliferation. To this aim a whole genome wide analysis of eNOS transduced HCASMCs was performed. A total of 19 genes were up regulated, and 31 genes down regulated in Ad-eNOS transduced HCASMCs compared to cells treated with an empty adenovirus. Noticeably, a cluster of HSP70 gene family members was amongst the genes up regulated. Quantitative PCR confirmed that transcripts for HSPA1A (HSP70A), HSPA1B (HSP70B) and HSPA6 (HSP70B') were elevated 2, 1.7 and 14-fold respectively in Ad-eNOS treated cells. The novel gene HSPA6 was further explored as a potential mediator of eNOS signaling in HCASMC. Immunoblotting showed that HSPA6 protein was induced by Ade-NOS. To functionally examine the effect of HSPA6 on SMCs, an adenovirus harboring the HSPA6 gene under the control of a constitutive promoter was generated. Transduction of HCASMCs with Ad-HSPA6 inhibited SMC proliferation at 3 and 6 days post serum growth stimulation, and paralleled the Ad-eNOS inhibition of SMC growth. The identification in this study that HSPA6 overexpression inhibits SMC proliferation coupled with the recent finding that inhibition of HSP90 has a similar effect, progresses the field of targeting HSPs for vascular repair.

Keywords: human vascular smooth muscle; nitric oxide; microarray; adenoviral gene transfer; heat shock protein 70.

Introduction

Cardiovascular disease is often associated with endothelial damage in the blood vessel wall. A consequence of the endothelial damage is the migration of smooth muscle cells (SMC) from the media to the intima layer of the arterial wall where they begin to proliferate. This stimulated growth of SMCs leads to the adverse narrowing of the lumen of the blood vessel, fibrous and ultimately restenosis. The endothelium in the blood vessel is the principal source of nitric oxide (NO), which is vital to suppressing SMC migration and proliferation [1,2]. One of the many pleitropic effects of NO is its inhibition on SMC proliferation. Vascular diseases including atherosclerosis, diabetes melitus and post-angioplasty restenosis are associated with a deficiency in NO production from the endothelium, which is thought to contribute to these disease states [3,4]. There are three main nitric oxide synthase (NOS) isoforms, nNOS (neuronal), iNOS (inducible) and eNOS (endothelial) and all catalyze the production of NO from L-arginine and oxygen. In the endothelium eNOS is the enzyme most active in generating NO, and whose deficiency is linked to vascular maladies [5,6]. It is established that reduced eNOS activity causes an increase in smooth muscle cell proliferation after vascular injury, leading to an increase in the intimal layer of the vascular wall, and further narrowing of the vessel lumen. Consequently, NOS gene therapy has been a promising therapeutic approach to vascular repair [7]. We and others have shown in rabbits that extraneous overexpression of eNOS by gene delivery to the vascular wall reverses thickening of the intimal layer by inhibiting SMC proliferation [8-10]. This phenomenon can be effectively mimicked *in vitro* by adenoviral delivery of an eNOS transgene into human arterial SMCs [11,12]. We and others have shown in this model that eNOS induces cell-cycle arrest by up regulating cell cycle regulators p27, p21 [11,13], and the tumour suppressor gene p53[14]. It is unknown if there are other targets of eNOS signaling, that may also be more amenable to therapeutic intervention. Given the importance of eNOS to vascular disorders it is surprising to date, that little is known about the mechanism by which eNOS/NO exert their effects on SMCs. To this end, we decided to examine the gene profile of eNOS transduced human coronary arterial smooth muscle cells (HCASMC) to further understand the influence of eNOS on smooth muscle cells. Here, we report the identification of a unique HSP70 gene family member called heat shock 70kDa protein 6 (HSPA6 or HSP70B'), which is induced by eNOS overexpression and can alone suppress smooth muscle cell growth.

Materials and Methods

Cell culture

Human coronary artery smooth muscle cells (HCASM) were grown according to manufacturer's instructions (Promocell). Briefly cells were grown in medium supplemented with fetal calf serum (5%), human epidermal growth factor (0.5ng/ml), human basic fibroblast growth factor (2ng/ml), human insulin (5µg/ml). Penicillin (100units/ml) and streptomycin (100mg/ml) were also added to the medium. Cells were grown in ventilated flasks at 37^{o}C in a humidified 5%CO_2, 95%O_2 incubator. Cells were passaged by trypsinization (0.04% trypsin/0.03% EDTA) and used between 3 and 7 passages.

Construction, Propagation and Purification of Adenoviral Vectors

An expression plasmid for HSPA6 in the pCMV-SPORT6 vector background was obtained from Open Biosystems,USA. This expression construct was recombined into the Donor vector of Invitrogen's Gateway system to generate an Entry vector according to manufacturer's instructions. The Entry vector construct was recombined into an adenoviral destination vector (pAd, Invitrogen, Carlsbad, CA) to generate the final adenoviral HSPA6 expression vector. Adenovirus was produced by transfecting purified Pac1 digested adenoviral expression vector into 293A cells and preparing a crude viral lysate. The crude lysate was used to infect 293A cells to amplify the virus. Adenovirus was purified by double cesium chloride (CsCl) gradient ultra-centrifugation. CsCl was removed by putting the virus suspension through a PD-10 column (GE Healthcare) with the final elution into Phosphate buffered saline (PBS). Viral stocks were stored at -80^{o}C and the viral titre determined by plaque assay. All other adenoviruses packaged with bovine eNOS (Ad-eNOS), a constitutively active form of human eNOS (Ad-eNOS S1179D) [15,16] or without a transgene (Ad-Null) were amplified and purified as above from previously used viral stocks [12].

Adenoviral Transduction

HCASMs were transduced as previously described [12]. Briefly cells were plated, and the following day transduced with viruses diluted in PBS with 0.5% albumin (PBS-A) at a multiplicity of infection (MOI) of 100 for 1 h. PBS-A was included as an additional control. The following day cells were transduced with adenoviral vectors encoding eNOS (Ad-eNOS), or empty vector (Ad-Null) at multiplicity of infection (MOI) of 100. Viruses were diluted in PBS with 0.5% albumin (PBS-A) and PBS-A was used as an additional control. Cells were exposed to the viral solutions for 1 hour following which medium was added.

Microarray Analysis

Microarray experiments were carried out using the Affymetrix 2 platform using the human array HGU-133A (Affymetrix High Wycombe, UK) which represents ~22,000 genes. HCASMCs were cultured, transduced as above and kept in medium for 48 hrs. Microarray analysis was carried out on RNA isolated from three separate treatments, Ad-eNOS, Ad-Null transduced and non-transduced HCSMCs and each treatment was done in triplicate (n=3) such that there were a total of 9 treatment samples for RNA purification and microarray analysis. RNA was isolated as described below. RNA quality was assessed by resolution of 28S and 18S ribosomal RNA by gel electrophoresis and microanalysis using the bioanalyser analysis system. Transgene expression, and no expression in control treatments were confirmed in each sample by RT-PCR of the eNOS transcript. Data analysis was performed using GeneSpring Version 7.2 from Silicon Genetics. Lists of interesting genes were isolated based on filtering by fold change. Only a fold change of at least two or more at a particular time point was considered as a significant change.

Real-Time PCR

RNA was extracted from cells transduced at a density of 1 x 10^6 on 100mm dish using Tri-reagent (Sigma) and an RNAeasy Kit (Qiagen) with DNase treatment according to manufacturer's instructions. RNA samples were quantified and reverse transcribed using the InProm-II Reverse Transcription System (Promega) and random primers. Quantitative polymerase chain reactions (Q-PCR) were each performed in a 15µl mixture containing 1-2µl of cDNA, 2x Fast SYBR Green Master mix (Applied Biosystems) and gene-specific PCR oligonucleotide primer pairs (Eurofins Genomics) using the standard heat-cycle parameters on the StepOne Plus PCR System (Applied Biosystems) and results analysed by StepOne Software v2.1. (PCR Primer details see Supplementary data Table 1 and Fig.1).

Western Blot Analysis of Transduced Cells.

Cells transduced at a density of 1 x 10^6 on 100mm dish were trypsinized, spun at 2000rpm, 5min and resuspended in lysis buffer (20mM Hepes pH 7.5, 1mM MgCl2, 350mM NaCl, 0.5mM EDTA, 1% NP40) with addition of a protease inhibitor cocktail (Pierce) and PMSF (Sigma). Cells were left on ice for 10min, mixed and spun at 13,000rpm for 5min. Aliquots were stored frozen and lysate protein was quantified by the BCA protein assay (Pierce).

Equal amounts of protein (20ug) were electrophoresed in 10% SDS-PAGE gel and transferred to a hybond nitrocellulose membrane. Membranes were blocked in 5% non-fat milk in Tris buffered saline, 0.1% tween (TBST) and probed with rabbit anti-HSPA6 (Abcam, 1:1000 dilution) and mouse anti-eNOS antibodies (BD Biosciences, 1:2000 dilution) respectively. Membranes were then incubated with horseradish peroxidase-conjugated anti-mouse-IgG and anti-rabbit-IgG respectively and visualized using enhanced chemiluminescent (ECL) detection (Amersham). Blots were reprobed without stripping with HRP-conjugated β-actin antibody (Abcam) to control for loading.

Immunofluorescent Analysis of cells.

Cells grown and transduced on sterile glass cover slips were fixed with 4% paraformaldehyde on ice for 5min, permeabilized with 0.05% Triton X-100, blocked in 10% fetal calf serum for 1 h, incubated with a rabbit anti-human HSPA6 antibody (Abcam) for 2 hrs, followed by secondary antibody Alexa fluor 594 conjugated IgG (Molecular Probes). Cells were mounted in an aqueous medium (Vector shield) with DAPI and viewed under Olympus BX51 upright fluorescent microscope.

Cell Counting

HCASMCs were plated and transduced the day after. Cells were then switched into serum free media for 3 days to synchronise cell growth. After 3 days and prior to serum stimulation, cells in one set of plates were counted and called day 0 in Fig.4B. The remaining cells were switched to media containing serum and allowed to grow. Cell counts after transduction prior to serum stimulation (Day 0) were similar among the four groups

Proliferation of cells was determined as previously described [12]. Briefly HCASMCs were plated in 6-well plates at 1 x 10^5 cells per well and transduced as above. After transduction 0.5% serum medium was added to cells for 48 hrs to render the cells quiescent to synchronize cell growth. After 48 hrs, growth was stimulated by adding 5% serum medium. Cells were counted on days 0, 3 and 6 using a Millipore Scepter handheld automated cell counter (Millipore) according to manufacture's instructions. For each day of cell counting, cells were washed with PBS, briefly trypsinized, spun, resuspended in 1ml of medium and counted. At each time point fresh medium was added to the remaining cells.

Statistical Analysis

Data is presented as mean ± standard error of the mean. Statistical analysis was performed by two tailed unpaired t-tests with significance at $p<0.05$.

Results

We performed a microarray analysis on adenoviral eNOS transduced HCASMCs after 48 hours. Fold differences were compared to both adenoviral null control cells and non-transduced cells. Before performing the microarray the eNOS transgene was confirmed to be overexpressed by RT-PCR analyses of the RNA isolated from transduced HCASMCs (data not shown). Microarray detected gene transcripts up-regulated above 2-fold in the eNOS transduced cells are summarized in table 1. The expression of 19 genes were up-regulated (>2 fold change) and 31 genes (>2 fold) were down-regulated in response to adenoviral-mediated overexpression of eNOS in HCASMCs compared to Ad-Null transduced cells and non-transduced cells (Table 1). The overexpression of eNOS was confirmed by detection of a 66-fold up-regulation of this gene on the microarray chip analysis of Ad-eNOS transduced HCASMCs (data not shown).

We validated select genes of interest from the microarray analysis including a cluster of HSP70 gene family members. To this aim, we performed quantitative real-time PCR on Ad-eNOS transduced HCASMCs compared to Ad-null transduced cells at 72 hours post transduction. As expected eNOS transduction inhibited the HCASMCs cell density after 72 hours as visible by light microscopy (Fig.1A). Gene expression levels at protein and mRNA were again confirmed by immunoblotting and Real-time PCR respectively, and levels were comparable to that achieved in the microarray experiments (Fig.1B,C). Real-time PCR of the eNOS transgene showed a 83-fold increase in eNOS transcript levels above Ad-Null controls at 72 hours (Fig.1C).

HSP70A and HSP70B transcripts were induced 2.14 and 1.7-fold respectively in Ad-eNOS versus Ad-Null treated smooth muscle cells (Fig.2A). In the same samples the human specific gene HSPA6 was detected to be induced 14.2 fold in Ad-eNOS compared to Ad-Null transduced HCASMCs (Fig. 2B). We wanted to ensure that the elevation in HSPA6 transcript was a specific response to eNOS *per se*. To this end, we further examined HSPA6 in non-transduced HCASMCs containing only the virus diluent, in Ad-Null transduced cells,

in Ad-eNOS transduced cells and in cells transduced with a constitutively active mutant (phosphomimetic) form of eNOS (S1179D). The adenovirus alone had no effect on HSPA6 compared to the PBS diluent (Fig.2C). Furthermore, Ad-eNOS and Ad-eNOS S1179D induced HSPA6 by 23.75 and 53.2-fold respectively above Ad-Null (Fig.2C). S1179D eNOS activated HSPA6 by 2.24 fold compared to wild-type eNOS, which parallels the two-fold increase in the rate of NO production from this mutant compared to wild-type eNOS [15]. In contrast, HSP70A and HSP70B were not further elevated by mutant eNOS S1179D and remained at similar levels to that induced by wild-type eNOS (Supplementary Fig. 2).

In order to confirm that elevated transcripts were concomitant to an increase in HSPA6 protein we performed immunoblotting of protein lysates prepared from Ad-eNOS and Ad-Null transduced HCASMCs respectively. The HSPA6 protein at 71kDa was specifically detected only in Ad-eNOS transduced HCASMCs. The antibody signal detection for HSPA6 was confirmed to be specific by the inclusion of protein lysates from HEK293 cells transfected with a HSPA6 expression plasmid and non-transfected cells respectively. HSPA6 protein expression was also seen to be further increased with the expression of the constitutively active Ad-eNOS S1179D compared to wild-type Ad-eNOS (Supplementary Fig.3).

In order to examine the function of HSPA6 we overexpressed the gene in HCASMCs. We attempted to transfect a plasmid encoding HSPA6 into HCASMCs by lipofection and electroporation, but levels of expression and cell viability were deemed insufficient for assessing the effects on cell function. Therefore, we generated an adenovirus harboring the HSPA6 gene under transcriptional control from the constitutively active cytomegalovirus (CMV) promoter. During the steps to generate the HSPA6 adenovirus (as described in methods) we wanted to confirm that the final adenoviral HSPA6 plasmid expressed HSPA6 protein. To this aim the adenoviral shuttle plasmid was transfected into HEK293 cells and we confirmed the 71kDa HSPA6 protein expression by immunoblotting (Fig.3A). Protein lysates from HEK293 cells with and without the original HSPA6 plasmid acted as positive and negative controls. The eventual generated purified adenovirus carrying the HSPA6 gene was then transduced into HCASMCs and expression confirmed by real-time PCR (Fig. 3B) and immunofluorescence (Fig. 3C). Real-time PCR robustly detected high fold change HSPA6 mRNA levels. HSPA6 protein was widely detected by immunofluorescence in HCASMCs. Noticeably, HSPA6 staining appeared distinctly punctate in the cytoplasm of all

smooth muscle cells. This may reflect the affinity of HSPA6 for subcellular localization within smooth muscle cells, as has been reported for HSPA6 in colon cells, [17] neurons [18], and macrophages [19] .

HCASMC Proliferation

HCASMCs transduced with Ad-Null, Ad-eNOS and Ad-HSPA6 respectively appeared normal, adherent and viable and on cell culture dishes when examined under bright-field microscopy compared non-transduced PBS-A treated cells (Fig. 4A). HCASMCs transduced with Ad-eNOS showed the expected decrease in cell counts compared to Ad-Null and non-transduced (PBSA) cells on day 3 and day 6 following stimulation with smooth muscle medium containing 10% FBS (Fig. 4B). Noticeably, Ad-HSPA6 showed a significant decrease in cell counts compared with Ad-Null and non-transduced PBS-A treated cells on day 3 and day 6. Interestingly, the decrease in cell number was greater in Ad-HSPA6 than in Ad-eNOS transduced cells, and compared to Ad-Null and non-transduced cells on day 3 and day 6. HSPA6 protein levels were detected by immunofluorescence staining of HCASMCs transduced with Ad-null, Ad-eNOS and Ad-HSPA6 respectively. HSPA6 was moderately detected in the Ad-eNOS transduced cells, with Ad-HSPA6 transduced cells exemplifying a more robust HSPA6 immunostaining, indicating higher protein expression (Fig 4B).

Discussion and Conclusions

In this study, in an effort to further understand the recognised inhibitory properties of eNOS and NO production on human SMC proliferation [11,14] [20], we have performed a genome wide gene expression analysis of eNOS transduced human vascular smooth muscle cells. Adenoviral eNOS transduction into SMCs is a model we and others have used to study the effect of localized delivery of NO to SMC [11,12,14,21], which is relevant to developing a gene therapy approach to halting deleterious intimal hyperplasia in injured arterial vessels [7] [6]. Following the validation of genes up and down-regulated, we identified a cluster of HSP70 genes that were up-regulated by eNOS overexpression in human SMCs. Amongst these genes was a unique HSPA6 gene, which we demonstrated to be highly up regulated by eNOS, and further elevated by a constitutively active form of eNOS. We also demonstrated that HSPA6 overexpression alone abrogated SMC proliferation in growth promoting cell culture conditions. This suggests that HSPA6 is a novel target of eNOS, which may be involved in mediating inhibition of human smooth muscle cell proliferation.

SMC proliferation is a confounding factor and contributor to vascular pathologies including disease states such as atherosclerosis and restenosis [22]. We and others have shown the efficacy of eNOS derived nitric oxide in inhibiting SMC proliferation [11,21] as well as in preventing in-stent restenosis [9]. During vascular remodelling after injury, eNOS is known to have a dual role of simultaneously stimulating re-endothelisation of the denuded vessel wall, whilst inhibiting the proliferation of SMCs, and hence neointimal re-growth [9,10]. For this reason eNOS is of major therapeutic interest for reasons of promoting vascular repair and prevention of in-stent restenosis after surgical intervention in atherosclerotic patients. Adenoviral delivery of NOS genes to the vasculature have shown promising results, with studies showing a reduction in intimal hyperplasia [8,12] [23]. We have shown that adenoviral delivery of eNOS accelerated re-endothelisation and inhibition of SMC proliferation in atheroclerotic rabbits [9,12]. We and others have also shown that eNOS can be delivered to arterial wall vessels by non-viral means using liposomes with efficacy in the form of endothelium regeneration [10,24] and inhibition of restenosis [10].

The events that lead to the eNOS driven inhibition of smooth muscle proliferation have been proposed to involve several factors including p27, p21 p53 [11] [14] and paxillin [23]. In the present study we identified three genes from the HSP70 family to be upregulated in response to eNOS overexpression including; HSP70-1A, HSP70-1B and HSPA6.

The HSP family are known to play a protective role when cells are subjected to stresses such as heat shock, toxins, heavy metals and radiation [25]. These cell stress proteins fulfill a wide range of functions, including cytoprotection, intracellular assembly, folding and translocation of oligomeric proteins [26]. HSP70 protein has been known to play a role in atherogenesis, although its precise role in cardiovascular disease remains unclear [25]. In the present study the observed upregulation of HSP 70-1A and -1B is consistent with a previous report showing NO inducing HSP 70 expression in SMC's via HSF 1 activation [27]. It is thought that the upregulation of HSP 70 is to protect the SMC's from injury as a result of NO stimulation [27].

The focus of our study was shifted to the observed more highly upregulated HSPA6 gene. HSPA6 is a unique human specific gene with no introns, with little known about its function [28]. HSPA6 is a strictly inducible member of the HSP 70 family with little or no basal

expression [29]. HSPA6 is closely related to HSP72 sharing 93% protein sequence homology and may have an overlapping function [17]. The findings in this study that a heat shock protein HSPA6 is induced by eNOS in SMCs and can inhibit smooth muscle proliferation is not unprecedented. Other HSP70 family members such as HSP70 has been shown to be protective against atherosclerosis and exogenous delivery of HSP70 has been proposed for clinical therapy [25].

There are multiple factors that can regulate vascular smooth muscle cells during atherosclerosis [30]. Some of these regulators can reduce atherosclerosis via preventing neo intimal hyperplasia. Relevant to the current study is the observation that heat treatment can inhibit neo-intima growth [31-33]. Interestingly, elevation in HSP72 (which has high homology with HSPA6) has been associated with the heat shock reduction in smooth muscle cell proliferation after trauma [31]. Two studies demonstrated that thermal treatment of arterial tissues *in vitro* and *in vivo* caused a reduction in media growth, leading to a prevention of arterial restenosis [33,34]. In one study hyperthermia at 43^{o}C was effective in suppressing rat vascular SMC proliferation in vitro by causing cell cycle arrest without causing cell death and not affecting endothelial cells which are normally damaged after angioplasty [34]. In the second study thermal treatment of cuff injured rat arteries in vivo inhibited neo-intimal thickening in association with increased HSP72 expression [33]. Therefore, heat shock proteins maybe obvious candidates to mediate this response. Since then several heat shock proteins have been postulated to be involved in atherosclerosis in a pro- or anti-atherosclerotic mode, although the mechanisms have not been clearly defined [25,35,36].

In this study HSPA6 was found to be upregulated 12.5 fold by eNOS overexpression in smooth muscle cells and expression of the constitutively active eNOS mutant further induced HSPA6 by 23 fold above controls (or ~1.8-fold above eNOS expression). The fold induction of HSPA6 closely parallels the known NO production from these two different eNOS transgenes with the constitutively active eNOS mutant producing double the amount of NO compared to wild-type eNOS [15]. Therefore, we would infer that HSPA6 is responding in a dose dependent manner to the NO generation by these differing transgenes. We further demonstrate that overexpressed HSPA6 alone can inhibit SMC proliferation. This suggests that HSPA6 is involved in the eNOS driven inhibition of SMC proliferation. Interestingly, in a separate study a DNA oligoarray analysis identified the HSPA6 gene to be strongly upregulated in Jurkat T cells treated with the NO releasing donar aspirin, which inhibits T-

cell proliferation [37]. Although the overexpression of HSPA6 in our study likely reaches supraphysiological levels, the findings nonetheless, indicate the therapeutic potential of HSPA6 as a target for diminishing intimal hyperplasia.

Notably, recent studies including Kim et al identified heat shock protein 90 involvement in atherosclerosis [38]. HSP90 is elevated in arterial plaques of atherosclerosis patients. Functionally, inhibition of HSP90 suppressed migration and proliferation of SMCs in human vascular SMCs, and in mice HSP90 blockage suppressed atherosclerotic plaque formation [38]. It was further deciphered that blockage of HSP90 reduced SMC migration via a down-regulation of integrinβ1, and matrix metalloproteinase-2. In addition, inhibition of HSP90 suppressed SMC proliferation via down-regulation of cyclinD3, proliferating cell nuclear antigen (PCNA) and retinoblastoma protein (pRb) leading to cell cycle arrest [38]. The Kim et al study is not unprecedented, as others have shown that targeted inhibition of elevated HSP90 in SMCs prevents intimal hyperplasia of injured mouse arterial vessels [39]. eNOS similarly induces cell cycle arrest in SMCs and has been shown to interact with HSP90 [40]. However, in endothelial cells (ECs) HSP90 associates with eNOS in promoting NO generation and inhibition of HSP90 in ECs causes uncoupling of eNOS, which decreases NO production [40] [41] [42]. Therefore, paradoxically the above studies suggest that HSP90 inhibition in EC versus SMC would have opposing effects on SMC growth in vivo.

Interestingly, HSP90 inhibition has been well documented in inhibiting cellular proliferation in various cancers and in the preparation of this manuscript a study was published that strongly demonstrates a large induction of HSPA6 upon HSP90 inhibition in cancer cells [43]. In light of our present study it will be interesting to examine the effect of nitric oxide on HSP90 and HSPA6 in human SMCs and if there is an interplay between these two heat shock proteins as has been reported in tumor cells.

The identification of eNOS targets has implications for understanding the mechanism for eNOS driven inhibition of smooth muscle cell proliferation and hence has implications as alternative therapeutic targets for the prevention of restenosis in coronary arterial disease. In summary we conclude that HSPA6 is a putative target of eNOS and is involved in the highly clinically relevant inhibition of smooth muscle cell proliferation. The findings from this study provide evidence to support the further investigation of a unique isoform of the HSP70 gene family in human atherosclerosis. HSPA6 could be an alternative target protein to modify by

gene therapy or pharmacology in preventing neointimal hyperplasia for vascular repair in human atherosclerosis as has been proposed for HSP70 [25] and more recently for HSP90 [38].

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LEGENDS

Fig.1

Effect of eNOS overexpression in human coronary arterial smooth muscle cells (HCASMCs). HCASMCs were transduced with adenovirus expressing bovine eNOS or adenovirus without transgene for 72 hours. (A) Representative bright field image of HCASMCs at 4x magnification post 72 hours transduction. Ad-GFP was transduced into HCASMCs to indicate the transduction efficiency of the adenovirus (B) Immunoblot for eNOS on protein lysates extracted from transduced HCSMCs. (C) Quantitative real-time PCR analysis of bovine eNOS from cDNA prepared from RNA extracted from transduced HCASMCs. Bar graph represents relative levels of eNOS transcript compared to housekeeping GAPDH transcript (bars; mean±SEM. n=3, three separate transductions measured in triplicate *$p<0.05$).

Fig.2

eNOS elevates Hsp70 genes and induces novel HSPA6 gene in HCASMCs. (A) Quantitative real-time PCR analysis of Hsp70A and Hsp70B transcripts in HCASMCs overexpressing eNOS. (B) Quantitative real-time PCR analysis of HSPA6 transcript in HCASMCs overexpressing eNOS. (C) Quantitative real-time PCR analysis of HSPA6 in Controls viral dilutant (PBS) and virus alone (AdNull) versus wild-type eNOS (Ad-eNOS) and a constitutively active mutant of eNOS (AdeNOS S1179D). (Bars; mean± SEM. n=3, from three separate transductions measured in triplicate *$p< 0.05$). (D) Representative immunblot for HSPA6, eNOS and loading control β-actin in eNOS overexpressing HCASMCs compared to Ad-Null control. Protein lysates from HSPA6 plasmid transfected and non-transfected HEK293 cells were used as a positive and negative controls for HSPA6 protein detection. Protein lysates were run from two separate viral transductions for each treatment.

Fig.3

Generation of of adenovirus to overexpress HSPA6 in HCASMCs. (A) Immunoblot detection of HSPA6 protein in HSPA6 adenoviral shuttle vector (pAd-HSPA6) transfection of HEK2993 cells used for preparing viral stocks. (B) Quantitative real-time PCR analysis of Ad-HSPA6 transduced HCASMCs post 24hours. (C) Representative immunofluorescent detection of HSPA6 in Ad-HSPA6 transduced HCASMCs. Nuclear DAPI staining appears as blue fluorescence and image appears at 40x magnification. Secondary antibody control stained cells were negative (not shown).

Fig.4

HSPA6 overexpression inhibits smooth muscle cell proliferation. (A) Representative bright field images of transduced HCASMCs on day 0, 3 and 6 post-transduction and replacement in full growth serum medium. (B) Immunofluorescent detection of HSPA6 levels in Ad-Null, Ad-eNOS and Ad-HSPA6 transduced HCASMCs (C) Automated cell count of transduced

HCASMCs on day 0, day 3 and day 6 (Bars; mean± SEM. n = 3, from three separate transductions with single count measurements, *$p<0.05$).

Supplementary Legends

Supplemental Fig. 1. Dissociation (melting) curves for all the genes were checked for the presence of primer dimers or spurious and not specific products. using 100 nM of each Primer (Listed in Suppl. Table1), and SybrGreen® PCR Master Mix. Reactions were amplified by 45 cycles of PCR using ABI StepOne Plus PCR System (Applied Biosystems). The melting curve analysis was performed by denaturation at 95°C for 15 sec, followed by a standard increasing ramp rate of the instrument from 60°C to 95°C. The X-axis is temperature and the Y-axis is Derivative or Fluorescence.

Supplemental Fig. 2. Quantitative real-time PCR analysis of HSP70A and HSP70B in virus alone (AdNull) versus wild-type eNOS (Ad-eNOS) and a constitutively active mutant of eNOS (AdeNOS S1179D). (Bars; mean± SEM. n=3, from three separate transductions measured in triplicate *$p < 0.05$).

Supplemental Fig. 3. Immunblot detection of HSPA6 on protein lysates from Ad-Null, Ad-eNOS and Ad-eNOS S1179D transduced HCASMCs. Protein lysates from duplicate transductions except for Ad-null are shown. Protein lysates from HSPA6 plasmid transfected and non-transfected HEK293 cells were used as a positive and negative controls for HSPA6 protein detection.

Genes up-regulated in response to eNOS overexpression
• Siah-interacting protein • chemokine (C-X-C motif) ligand 11 • haem oxygenase (decycling) 1 • DnaJ (Hsp40) homolog, subfamily B, member 6 • DnaJ (Hsp40) homolog, subfamily A, member 1 • phorbol-12-myristate-13-acetate-induced protein 1 • cysteine and histidine-rich domain (CHORD)-containing, zinc binding protein 1 • heat shock 105kDa/110kDa protein 1 • neuregulin 1 • crystallin, alpha B • heat shock 105kDa/110kDa protein 1 • DnaJ (Hsp40) homolog, subfamily B, member 1 • interleukin 7 receptor • heat shock 70kDa protein 6 (HSP70B') • heat shock 70kDa protein 1A • heat shock 70kDa protein 1B • heat shock 70kDa protein 1A • zinc finger protein 236 • endothelial nitric oxide synthase

Genes down-regulated in response to eNOS overexpression
• EGF-containing fibulin-like extracellular matrix protein 1 • GATA binding protein 6 • KIAA1199 protein • nuclear factor I/B • dihydropyrimidine dehydrogenase • forkhead box G1B • fibroblast growth factor 2 (basic) • aldehyde dehydrogenase 1 family, member A3 • leukocyte-derived arginine aminopeptidase • ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin) • fibroblast growth factor 7 (keratinocyte growth factor) • fibrillin 2 (congenital contractural arachnodactyly) • solute carrier family 4, sodium bicarbonate cotransporter, member 4 • platelet-derived growth factor receptor, alpha polypeptide • cytochrome P450, family 1, subfamily B, polypeptide 1 • HEG homolog • heterogeneous nuclear ribonucleoprotein A3

- retinoic acid receptor responder (tazarotene induced) 1
- antigen identified by monoclonal antibody MRC OX-2
- mesoderm specific transcript homolog (mouse)
- collectin sub-family member 12
- cytochrome P450, family 1, subfamily B, polypeptide 1
- topoisomerase (DNA) II alpha 170kDa
- growth arrest-specific 1
- pentaxin-related gene, rapidly induced by IL-1 beta
- SRY (sex determining region Y)-box 9
- HEG homolog
- latrophilin 2
- recombining binding protein suppressor of hairless (Drosophila)
- desmoplakin
- chromosome 9 open reading frame 26 (NF-HEV)

Table 1. Genes up-regulated and down-regulated (>2 fold) in response to adenoviral-mediated overexpression of eNOS in HCASMCs compared to adenovirus null controls.

FIG.1

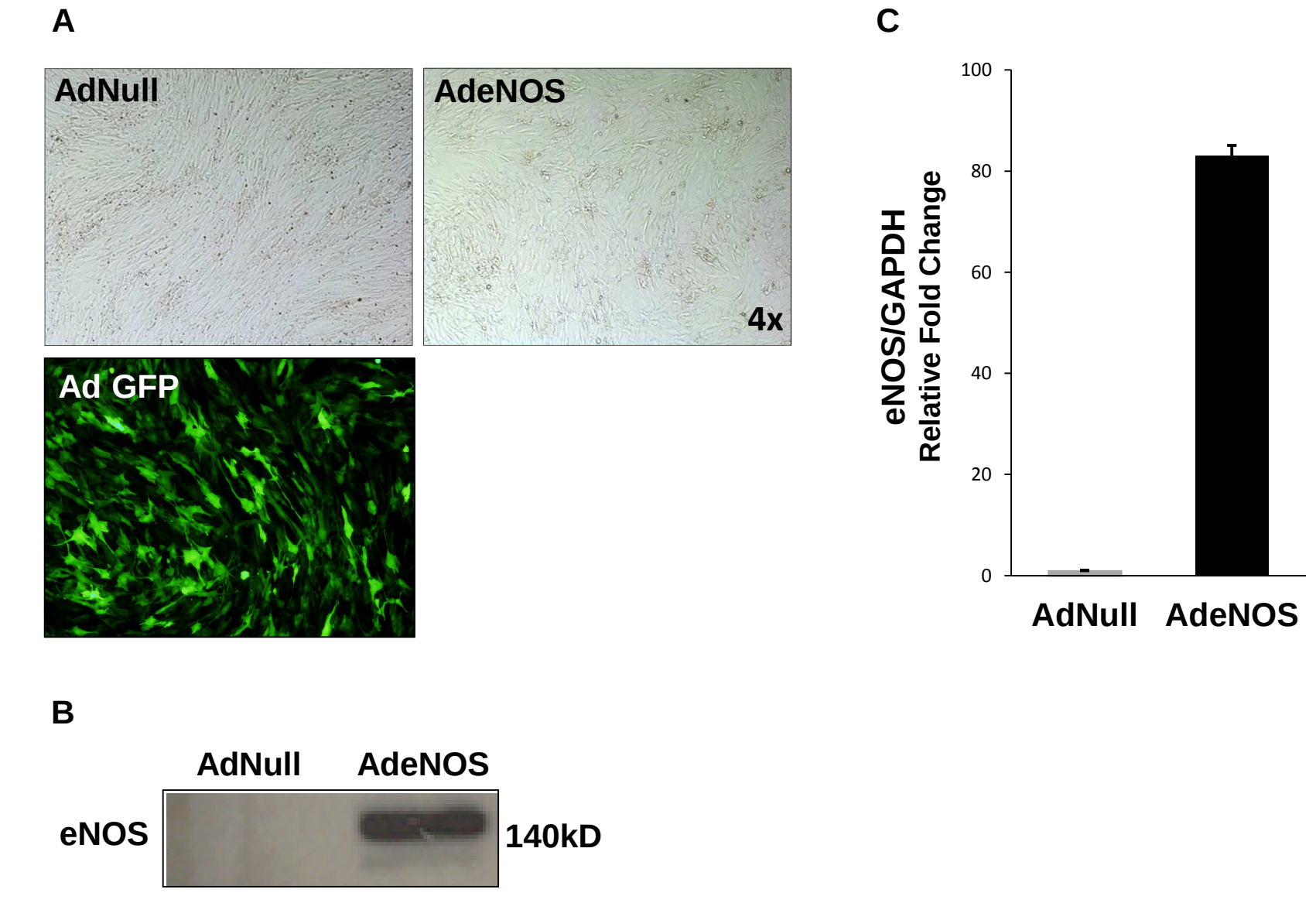

A
AdNull
AdeNOS
4x
Ad GFP
C
eNOS/GAPDH
Relative Fold Change
100
80
60
40
20
0
AdNull
AdeNOS
B
AdNull
AdeNOS
eNOS
140kD

FIG.2

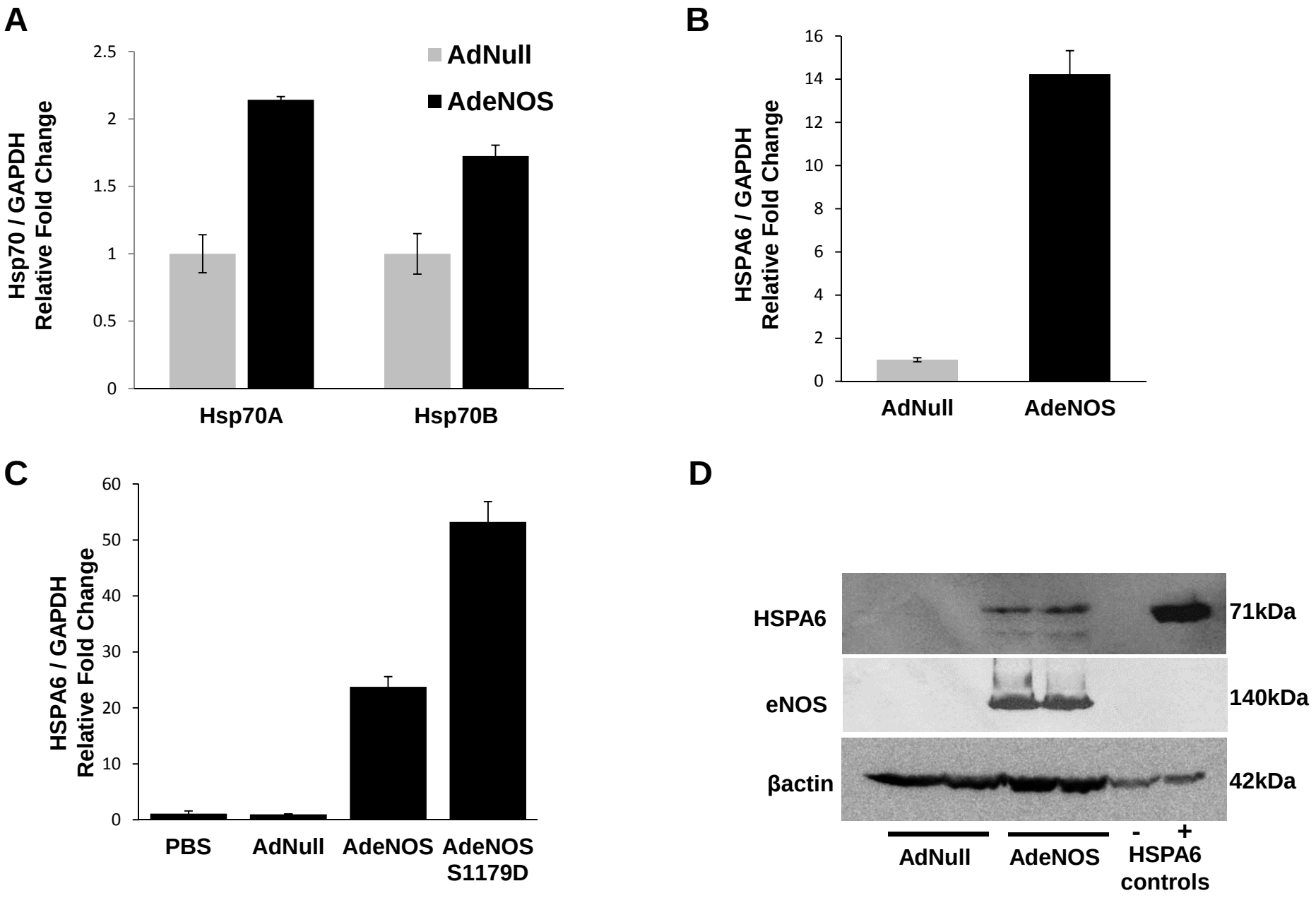
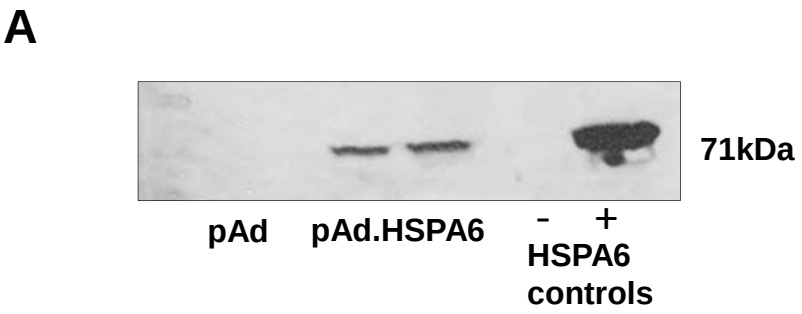
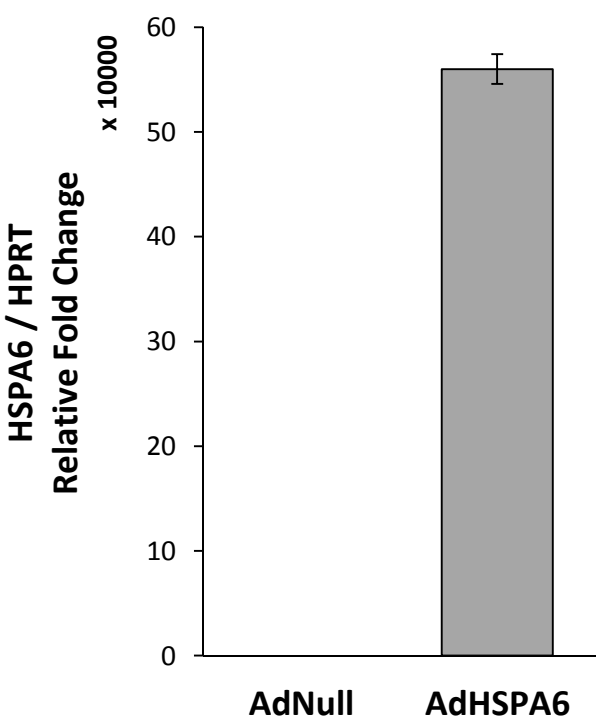

A
Hsp70 / GAPDH
Relative Fold Change
2.5
2
1.5
1
0.5
0
AdNull
AdeNOS
Hsp70A
Hsp70B
B
HSPA6 / GAPDH
Relative Fold Change
16
14
12
10
8
6
4
2
0
AdNull
AdeNOS
C
HSPA6 / GAPDH
Relative Fold Change
60
50
40
30
20
10
0
PBS
AdNull
AdeNOS
AdeNOS S1179D
D
HSPA6
71kDa
eNOS
140kDa
βactin
42kDa
AdNull
AdeNOS
-
+
HSPA6 controls

FIG.3


A
71kDa
pAd
pAd.HSPA6
-
+
HSPA6
controls


B
HSPA6 / HPRT
Relative Fold Change
x 10000
60
50
40
30
20
10
0
AdNull
AdHSPA6

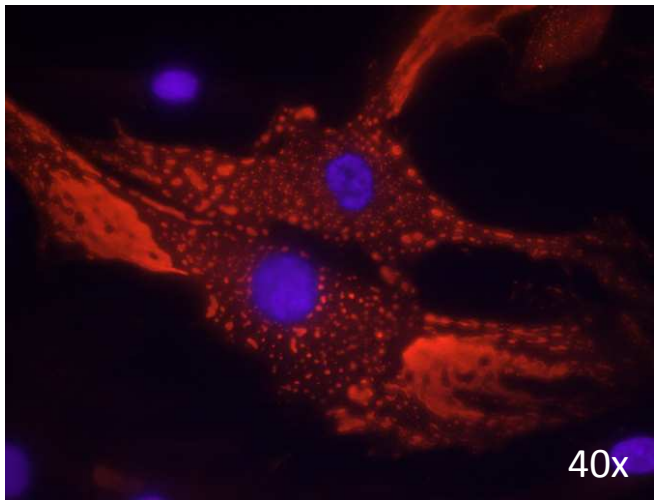

C
40x

FIG.4

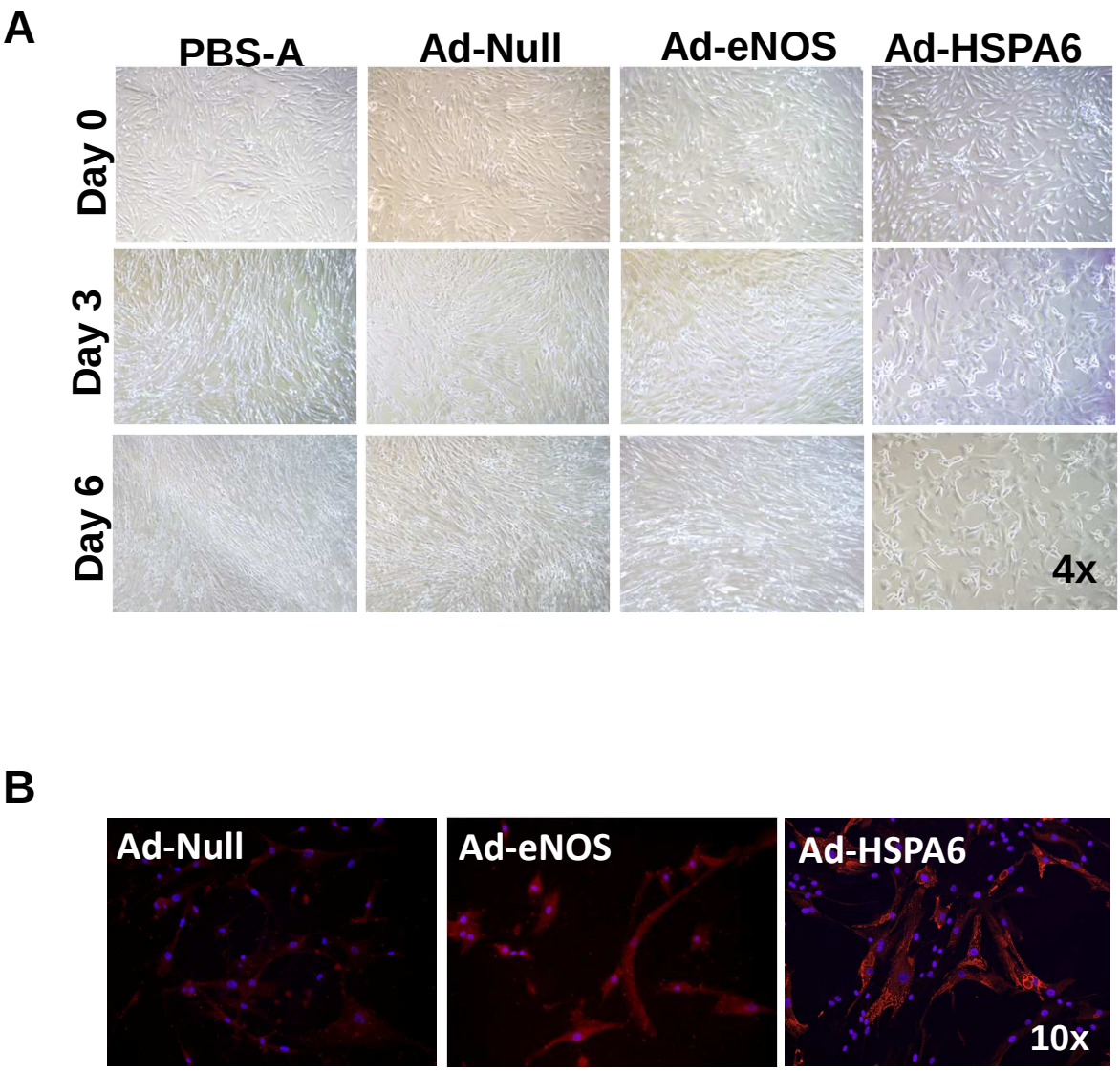
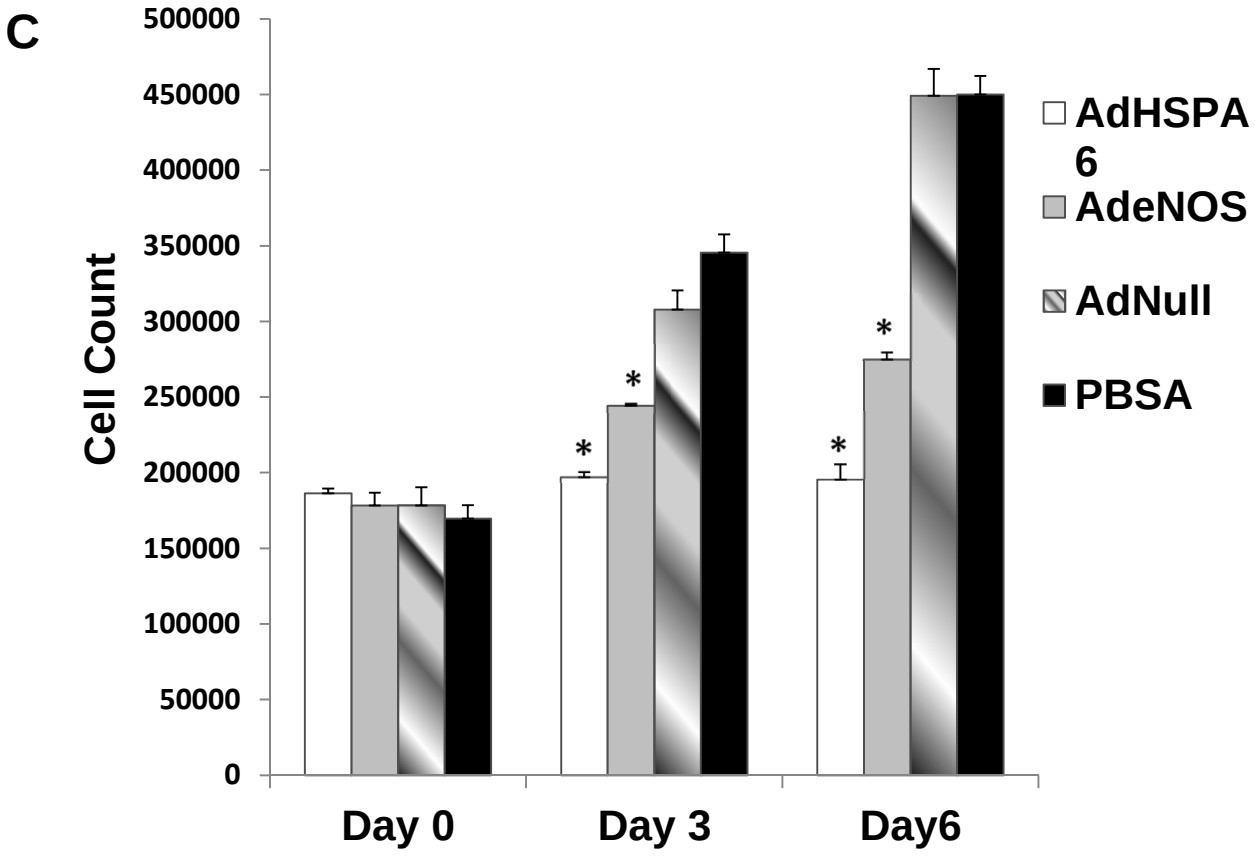

A
PBS-A
Ad-Null
Ad-eNOS
Ad-HSPA6
Day 0
Day 3
Day 6
4x
B
Ad-Null
Ad-eNOS
Ad-HSPA6
10x

FIG.4


C
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
Cell Count
Day 0
Day 3
Day6
AdHSPA 6
AdeNOS
AdNull
PBSA
*
*
*
*

Highlights

- Whole genome wide screen of eNOS regulated genes in human arterial smooth muscle cells was performed in search of novel eNOS/NO sensitive genes.
- Adenoviral overexpression of eNOS induces heat shock HSPA6 (or HSP70B') at transcript and protein level in human coronary arterial smooth muscle cells
- HSPA6 induction responds to eNOS transgenes with increasing enzymatic activity for nitric oxide production.
- Overexpression of HSPA6 potently inhibits human coronary arterial smooth muscle cell proliferation.