Genetic Induction of a Releasable Pool of Factor VIII in Human Endothelial Cells

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Abstract—Although it is known that factor VIII (FVIII) plasma levels increase rapidly in response to a number of stimuli, the biological stimuli behind this release is less clear. Previously, we showed that FVIII can traffic together with von Willebrand factor (vWF) into storage granules in a pituitary tumor cell line, AtT-20; however, AtT-20 cells could not be used to address the release or functional activity of released FVIII. To investigate the regulated secretion of stored FVIII, endothelial cells with intact agonist-stimulated release pathways were used. Human umbilical vein endothelial cells (HUVECs) were transduced with retroviral FVIII construct [hFVIII(V)] to create a FVIII/vWF storage pool. Immunofluorescent staining of transduced cells demonstrated FVIII in Weibel-Palade bodies. In contrast, the transduction of hFVIII(V) into HT-1080 and HepG2 cells displayed FVIII only in the cytoplasm. We studied the regulated release of both FVIII and vWF from endothelial cells after agonist-induced stimulation and demonstrated a parallel release of FVIII and vWF proteins. This released FVIII was functionally active. Hence, endothelial cells transduced with hFVIII(V) store FVIII together with vWF in Weibel-Palade bodies, creating a releasable storage pool of both proteins. Because FVIII secretion can be physiologically regulated by agonists in culture, this may explain the pharmacological agonist-induced release of FVIII by drugs such as desmopressin in vivo and suggests vascular endothelium as a reasonable target of gene therapy of hemophilia A. (Arterioscler Thromb Vasc Biol. 2000;20:2689-2695.)

Key Words: factor VIII ■ von Willebrand factor ■ endothelial cells ■ agonist stimulation ■ hemostasis

Hemophilia A is an X-linked disorder caused by an absence of or a reduction in coagulation cofactor protein, factor VIII (FVIII). Normally, FVIII circulates as a noncovalent complex with its carrier protein, von Willebrand factor (vWF); an interaction that protects FVIII from proteolytic degradation. Little is known about whether they first interact intracellularly or extracellularly. Although there is good evidence that FVIII is synthesized in the liver,2,3 it is not the sole source of FVIII. FVIII mRNA has been detected in multiple organs, including the spleen and lymph nodes. Organ transplantation studies in recent decades have identified that the liver,4 as well as several extrahepatic sources of FVIII (lungs, lymphatics, and spleen),5-7 produces FVIII in humans and dogs with hemophilia A. Although FVIII synthesis by hepatocytes has been demonstrated,8 end-stage liver disease is not associated with a decrease in plasma FVIII levels.9 Even within the liver, the cell type or types in which the FVIII is synthesized are not clearly established.8,10

The synthesis of vWF has been demonstrated in endothelial cells and megakaryocytes, where it is stored in specialized vesicles: Weibel-Palade bodies (WPBs) and α-granules, respectively.11,12 In the late trans-Golgi network, vWF is sorted into releasable dense-core granules.13 These endothelial granules consist of mature vWF; its propeptide, vW-AgII; and 2 receptor proteins, P-selectin and lamp 3.11 In certain tissues, the endothelial cell WPBs can also contain interleukin (IL)-8.14 A large number of external stimuli activate granule release (exocytosis) from the endothelium, including mediators of inflammation and hemostasis and vasoactive drugs such as 1-desamino-8-D-arginine-vasopressin (DDAVP; desmopressin).15,16 The administration of DDAVP to normal individuals causes a rapid and parallel increase of FVIII and vWF into plasma.17,18 Although endothelial cell WPBs appear to be the source of this DDAVP- and epinephrine-induced release of vWF,19 the source of the released FVIII has not been determined, nor have FVIII storage sites been identified in tissues. By creating a model system to mimic these processes, our aim was to better understand the cellular biology involved in the establishment of FVIII storage and release.

Previously, we established that in transfected murine neuroendocrine cells (AtT-20), the subcellular localization of FVIII is altered by vWF trafficking, resulting in the storage of both proteins within the same granule.20 Although AtT-20

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cells can store both vWF and FVIII, the regulated release of FVIII and the maintenance of its functional integrity after release required study in a more physiologically relevant cell. Primary endothelial cells permitted us to create a model that could address the regulated release of FVIII from native storage granules and to determine whether FVIII remained functionally active after the release of both vWF and FVIII from a storage pool.

Methods
Supplemental material can be viewed at http://atvb.ahajournals.org.

Transduction of Retroviral Constructs
The retroviral vector hFVIII(V) used in these experiments contains a B-domain deleted (BDD) FVIII cDNA and was produced in a human packaging cell line by Chiron Corporation. The retroviral vector used in these experiments had a titer of 2.6 × 10^6 cfu-eq/mL. All cells were seeded at 2 × 10^5 cells per well and grown until ∼50% confluent. Transduction conditions were optimized at a multiplicity of transduction (MOI) of 80, with the viral vector added directly to the cultures at 24 and 48 hours. Conditioned media were harvested from confluent cells at 120 hours after transduction. Polybrene (1 mg/mL) was added to inactivate heparin, and the media were centrifuged at 14,000 rpm to remove cellular debris. The supernatants were frozen at −80°C until assayed.

Agonist Stimulations
The acute release of FVIII and vWF in conditioned medium by PMA, ionomycin, histamine, TRAP, leukotriene (LT)C₄, forskolin, and isoproterenol was monitored after agonist-induced stimulation of the endothelial cells. Confluent cell monolayers were pretreated with cycloheximide (5 μg/mL) diluted in complete DMEM/HUVEC for 6 hours (37°C) to arrest protein synthesis. The cells were washed twice with HBSS and incubated with 0.05 μL complete DMEM/HUVEC for 30 minutes at 37°C before agonist stimulations to determine the constitutive release levels of FVIII and vWF (sham incubations). The cells were treated with various chemical and physiological agonists to induce the release of vWF, FVIII, or both. Agonist stocks were dissolved in DMSO (forskolin, IBMX, ionomycin, isoproterenol, and PMA), sterile water (amastatin, histamine, thrombin, and TRAP), or methanol (LTC₄), and then each was diluted in complete DMEM/HUVEC. The final concentration of DMSO in the stimulation medium did not exceed 0.2%. The cells were incubated at 37°C for 30 minutes in the presence of agonists or inhibitors (1 μmol/L PMA, 1 μmol/L ionomycin, 10 μmol/L histamine, 60 μmol/L TRAP plus 80 μmol/L amastatin, 50 nmol/L LTC₄, 25 μmol/L forskolin plus 1 nmol/L IBMX, and 10 μmol/L isoproterenol plus 1 nmol/L IBMX) based on published conditions. Negative controls included with each set of agonist stimulations were media alone, media plus 0.2% DMSO, or non-treated confluent cells. After agonist treatments, conditioned media were harvested and stored as described earlier. The cells were then formalin fixed and preserved for later immunofluorescent detection of subcellular antigens.

Indirect Immunofluorescence
After the harvest of conditioned medium, the cells were fixed, permeabilized, and immunostained with a sequential series of antibodies and examined in detail with confocal laser scanning microscopy, as previously described. In the majority of the staining reactions, anti-FVIII monoclonal antibody (MBC 103.3) was paired with a rabbit anti-vWF polyclonal antibody to detect the localization of the 2 proteins. In addition, each set of immunostained cells contained both positive and negative antibody controls in separate wells.

Activity and Antigen Assays
FVIII activity (FVIII:C) and v-WF antigen (vWF:Ag) levels in the 24-hour and 30-minute harvested samples were quantified by both functional (FVIII Coatest VIII:C/4 kit; Chromogenix) and immunological (vWF ELISA) assays, as previously described. All samples were measured in triplicate. Standards for these assays were purified rh-BDD-FVIII (ReFacto; Pharmacia) or normal human pooled plasma diluted in media, with normal plasma levels defined as 1 U/mL FVIII or vWF.

Data Analysis
The raw data (mOD/min and OD) were converted into concentrations (mU/mL) through the use of a standard curve that was generated for each assay plate. Experiments were repeated a minimum of 3 times, and the results were averaged. The amount of FVIII or vWF released by stimulation is defined as the amount of FVIII or vWF in the conditioned medium with agonist minus the concentration of FVIII or vWF in the conditioned medium from preincubation without agonists. The quantity of vWF and FVIII measured varied from experiment to experiment; therefore, the results were normalized to the PMA agonist-stimulated release data, these values were normalized to the PMA agonist-stimulated release as measured in the same experiment (“% Maximal”). Results from the different replicates performed in separate experiments were then averaged on the basis of the “% of maximal release” from each experiment. The absolute values of the PMA-induced acute release are given in the figure legends for relevant experiments.

Results
FVIII Expression in Transduced Primary Endothelial Cells
To investigate the storage and secretion of FVIII in human tissue, we performed preliminary studies to address the capability of endothelial cells to express FVIII. After hFVIII(V) transduction of primary HUVECs, FVIII:C levels in the conditioned medium ranged from 453 to 642 mU · mL⁻¹ · d⁻¹ · 1 × 10⁶ cells (~ 50% normal plasma levels). With this retroviral vector and optimal transduction conditions, the number of immunodetectable FVIII-expressing cells was ∼50% to 60% of total cells as viewed with epifluorescence microscopy. In non-vWF-expressing cells such as HT-1080 fibroblasts under similar conditions, the levels of FVIII:C were elevated but to a lower amount after retroviral transduction (from 0 to 251.7 ± 25.1 mU/mL · d⁻¹ · 1 × 10⁶ cells). We detected insignificant differences in the amount of vWF expressed by the nontransduced and transduced HUVECs 30.06 ± 3.37 versus 28.02 ± 5.92 mU vWF:Ag · mL⁻¹ · d⁻¹ · 1 × 10⁶ cells, respectively) as measured with the vWF ELISA (Figure 4), demonstrating that vWF expression and release were not affected by the presence of the retroviral constructs.

Expressed FVIII Is Trafficked to WPBs
The subcellular location of expressed FVIII was determined by immunostaining the transduced HUVECs with several different antibodies. As shown in Figure 1, in transduced cells FVIII was observed intracellularly both as intense staining vesicles scattered throughout the endothelial cells and as diffuse perinuclear staining (Figure 1A). The endogenous vWF was detected in every endothelial cell on the slides as dense staining granules (Figure 1B). When compared in the merged images (Figure 1C), all FVIII granules align with corresponding vWF granules (shown in yellow). This storage pattern of FVIII in HUVECs was in direct contrast to that observed in non-vWF-expressing cells, such as the epithelial cell line HT-1080. There were no dense aggregates of FVIII present in the retrovirally transduced HT-1080 cells (Figures 1D to 1F). Moreover, when transduced HUVECs were stained for FVIII and v-WF-II or P-selectin, granular stored FVIII (online Figures IB and IE) was noted to be colocalized with both proteins (online Figures IC and IF). Detection of
endogenously synthesized FVIII in nontransduced HUVECs was not observed with confocal microscopy or detected with functional assays of conditioned media. Furthermore, although secreted FVIII levels in the transduced cultures were high (453 to 642 mU/mL), endocytic uptake of this secreted FVIII did not occur in cultured HUVECs, as denoted by the lack of FVIII detection in the cells surrounding the immunoreactive HUVECs (≤50% of cells).

Activation of the WPBs and Release of Stored FVIII/vWF

To determine whether the stored FVIII was both releasable and activatable, we assembled a panel of agonists that are known to induce release of WPBs. This allowed confirmation that nascent FVIII/vWF WPBs remain responsive to a wide range of secretagogues. Agonist-stimulated release studies were performed after a 6-hour pretreatment with cycloheximide to avoid de novo protein synthesis or the accumulation of nascent, constitutively secreted proteins. Agonist-stimulated release studies were performed after a 6-hour pretreatment with cycloheximide to avoid de novo protein synthesis or the accumulation of nascent, constitutively secreted proteins. Agonist stimulation of the transduced HUVEC cultures increased the vWF and FVIII levels in parallel: 3- to 7-fold (for FVIII) and 3- to 13-fold (for vWF) over that of nonstimulated (media only) cultures (Figure 2). The agonists that stimulates the largest increases in FVIII and vWF under our experimental conditions were PMA (7-11-fold for FVIII/vWF) and the mixture of forskolin/IBMX (5.5-12.5-fold for FVIII/vWF). The average release for FVIII/vWF after stimulus with ionomycin, histamine, LTC4, and forskolin was 37.2/52.5%, 57.8/64.4%, 34.3/41.4%, and 56.1/118%, respectively (Figure 2, lanes 5/6, 7/8, 11/12, and 13/14, respectively). The majority of agonists tested led to significant release of FVIII and vWF.

Visual analysis of WPB migration with indirect immunofluorescent microscopy confirmed that exocytosis of FVIII/vWF granules had occurred in response to agonist stimulation. As in the earlier experiments performed on transduced HUVECs (Figure 1 and supplement I), the agonist-treated cells were fixed and immunostained for FVIII and vWF and examined through confocal microscopy. This analysis showed the expected “release patches” that consisted of fused WPBs as noted by previous investigators and an overall loss of granule numbers (Figures 3G to 3I and 3J to 3L), indicating that induced exocytosis of the WPBs had occurred. Nonstimulated control experiments displayed no WPB loss in either the nontransduced or the retrovirally transduced HUVECs (Figures 3A to 3C and 3D to 3F). The agonists that generated the highest release levels (PMA and forskolin) showed the highest degree of intracellular granule depletion.
The degree of WPB depletion varied between the different agonists (25% to 90% of WPBs in the cells) and between cells in each culture, where some cells appeared to be unaffected by the agonists.

**FVIII Expression in Other Cells**

Having illustrated that FVIII is expressed after transduction and can be stored with vWF in HUVECs, we next explored FVIII expression in other isolated vascular endothelial and hepatic cells. Recent studies by Yamamoto et al. that examined the distribution of vWF in murine tissues showed that the lung and brain possessed the highest vWF mRNA levels and that the thoracic aorta, as well as the arteries and microvessels of the lung, displayed high expression of vWF antigen. We obtained isolated endothelial cells from human lung microvascular beds (HMVEC-Ls) and pulmonary arterioles (HPAECs) to compare endogenous production of vWF and FVIII to that of bovine aortic endothelial cells and human immortalized hepatocytes (HepG2). Separate cultures of each cell type were transduced with the retroviral FVIII vector under the same conditions as for the HUVEC transductions. Conditioned media samples were analyzed for FVIII:C activity and vWF:Ag levels (Figure 4) and compared with those of primary HUVEC transductions. The secreted vWF antigen levels from the various endothelial cell types were similar in both the nontransduced and transduced cells (filled columns 1 to 4) and appear similar to that expressed by primary HUVECs (an average of 8.5 versus 30 mU/mL vWF:Ag). In the absence of transduction (Figure 4, cross-hatched columns 1, 3, 5, and 7), the FVIII:C activity levels measured were below the limits of the detectable range of the assay (6 mU/mL FVIII:C). After transduction of the retroviral FVIII vector into the endothelial cell subtypes, a dramatic increase was observed in secreted FVIII:C levels (Figure 4, cross-hatched columns 2, 4, and 6). In fact, the levels of secretion of FVIII:C in the HPAEC transductions were twice that seen in the HUVEC experiments (1159±63 versus 548±85 mU/mL FVIII:C · d⁻¹ · 1×10⁶ cells). Compared with that of the transduced endothelial cells, FVIII expression in retrovirally transduced HepG2 cells was substantially lower. Only a small amount of FVIII secretion could be detected (28 mU/mL) (Figure 4, column 8), representing a 25- to 40-fold decrease in FVIII:C media levels compared with that of endothelial cells.

Indirect immunofluorescent staining was performed on these cultured endothelial cell lines that confirmed FVIII storage within the WPBs. The cells were examined with anti-FVIII and -vWF antibody mixtures (Figure 5). In the nontransduced endothelial cells, the characteristic long-rod WPBs are clearly evident in every cell when stained with anti-vWF antibodies (Figures 5A and 5C); however, when stained with anti-FVIII antibodies, no FVIII was observed in the native cells (Figures 5A, 5C, and 5E). This correlates with the negative results observed in the FVIII functional assays (Figure 4). On retroviral FVIII transduction, FVIII is produced and cistored with vWF in granules (Figures 5B and 5D). In contrast, there were no storage deposits detected in the transduced HepG2 cells (Figure 5F), only a faint diffuse cytoplasmic staining. The lack of FVIII storage in the hepatic cell line is analogous to the patterns seen with the transduced fibroblast cells, as depicted in Figures 1D to 1F). Although no endogenous expression of FVIII could be detected in various endothelial cells, FVIII synthesis appears to be readily induced in retrovirally transduced cells.
that high levels of FVIII expression are possible. FVIII construct of these endothelial types clearly demonstrates native lung microvascular and pulmonary arterial endothelial axis with a reduced scale. Although FVIII expression in cultured curves were plotted along the $x$-axis with a reduced scale. Although FVIII expression in cultured

If $x$-axis labels. The concentration of FVIII and vWF is expressed as $\text{mU/mL (\pm SEM, n=3)}$. To show both FVIII and vWF results on the same chart, the FVIII:C curves are plotted along the $x$ axis using the (left) y axis, whereas the vWF:Ag curves were plotted along the $x$ axis using a second (right) $y$ axis with a reduced scale. Although FVIII expression in cultured

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Discussion

DDAVP is known to trigger a rapid concomitant increase in both FVIII and vWF.\textsuperscript{17} The mechanism by which this physiological pool of FVIII is formed is unknown.\textsuperscript{18} Previously, we demonstrated that vWF could influence FVIII biosynthesis by altering its intracellular trafficking so that the 2 proteins were stored together in AtT-20 cells.\textsuperscript{20} Whether this FVIII was releasable and whether it was functionally active could not be determined in AtT-20 cells because they have a known variable response in their release of stored proteins.\textsuperscript{11} To circumvent this problem, we extended our studies to normal human endothelial cells so the release and functional activity of FVIII could be determined. Vascular endothelium has been widely studied for its regulated release of stored proteins (vWF, IL-8, and tPA).\textsuperscript{13,14,32} We now have been able to demonstrate the regulated release of FVIII together with vWF and that the released FVIII is functionally active.

To establish FVIII expression in human endothelium, we transduced primary HUVEC isolates with a retroviral FVIII construct, hFVIII(V), that resulted in FVIII production that was nearly 200-fold greater than the levels we had previously obtained with plasmid FVIII in transfected AtT-20 cells.\textsuperscript{20} During a 24-hour period, 1 million cells produced 548 to 1159 mU·mL$^{-1}$·d$^{-1}$, and the medium contained active FVIII that was $\approx$80% of normal plasma levels. Because the human body contains 1 to 6×10$^{13}$ endothelial cells,\textsuperscript{33} FVIII gene therapy would require that only 1 in 200 000 cells be stably producing FVIII to establish a minimal therapeutic levels of 5 U/dL. The additional amount of FVIII stored in secretory granules could account for even greater levels either locally or systemically after physiological or pharmacological stimulation.

FVIII expressed in endothelial cells trafficks and colocalizes with vWF in storage granules similar to AtT-20 cells\textsuperscript{20} (Figure 1 and supplemental Figure 1). In contrast, hFVIII(V) transduction of non–vWF-expressing cells (HT-1080 and HepG2) produced only diffuse staining of immunoactive FVIII throughout the cytoplasm (Figures 1D and 5F) and no detectable storage of FVIII. This further demonstrates that storage of FVIII does not occur in the absence of vWF. To establish whether FVIII was only being stored in WPBs, HUVECs were immunostained for other WPB components (vW-AgII and P-selectin), and both colocalized with stored FVIII (supplemental Figure 1). We therefore conclude that FVIII is stored in WPBs of endothelial cells when synthesized endogenously in these cells.

The biosynthesis and storage of vWF in endothelium represent a storage pool of synthesized vWF protein that can be released systemically or locally after physiological stimuli.
or vascular damage. A wide variety of agents have been demonstrated to mediate the release of this vWF storage pool, including thrombin (hemostasis), histamine and leukotrienes (inflammation), and epinephrine and DDAVP (vasoactive agents), with the release occurring within minutes after administration.\textsuperscript{15,16} This release correlates with the activation and fusion of the endothelial cell WPBs with the plasma membrane (exocytosis).\textsuperscript{30} Nine agonists were chosen to induce the activation pathways of HUVECs (see Figures 2 and 3). When FVIII was coexpressed in endothelial cells, agonists released both vWF and FVIII into the condition media. The greatest response occurred after PMA activation and occurred, to a lesser degree, due to forskolin, histamine, and LTC\textsubscript{4} stimulation (Figure 2). We would expect FVIII expressed in endothelium to undergo the same physiological and pharmacological release that has been demonstrated at many laboratories for vWF.\textsuperscript{22,24,27,34} Figure 3 demonstrates visually that granular deposits of FVIII and vWF are released in response to agonist stimulation and not by cell lysis. No change in intracellular localization of FVIII was seen when transduced fibroblasts were studied.

Heterogeneity exists between vascular beds and leads to differences in local production of cytokines and coagulation proteins (tPA and vWF).\textsuperscript{31,33,35,36} The chemokine IL-8 is variably expressed in endothelial cells, colocalizes with vWF in the WPBs, and is released on agonist stimulation.\textsuperscript{14,37} Isolated endothelial cells from multiple sources, including the microvascular bed of the lung and the pulmonary artery, produced vWF, but FVIII was detected only if these cells were transduced with FVIII cDNA (Figures 4 and 5). Because FVIII synthesis after transduction with hFVIII(V) was much greater in endothelial cells than in similarly transduced fibroblasts or hepatocytes, one explanation would be that the cosynthesis of vWF permitted more efficient intracellular synthesis of FVIII, similar to that identified by Kaufman et al.\textsuperscript{38} in Chinese hamster ovary cells and more recently in vWF-deficient pigs.\textsuperscript{39} Furthermore, a report from Do et al\textsuperscript{10} has shown that murine hepatic sinusoidal endothelial cells possess the ability to express FVIII.

Although the synthesis of FVIII by endothelial cells would help to explain the FVIII storage pool that is released by DDAVP, the identification of the cell type or types that physiologically synthesize FVIII awaits further study and clarification. Perhaps the more important issue is whether gene therapy that directs FVIII synthesis to the endothelium might be a more efficient means of achieving effective synthesis and processing of FVIII. Such synthesis would establish a protected, releasable pool in endothelial cells and be under the potential pharmacological control of intranasal DDAVP. Whether the induction of FVIII synthesis by cells other than endothelium can result in FVIII trafficking to endothelial vWF storage sites remains to be determined. Current protocols for in vivo gene therapy may limit FVIII synthesis to the liver by placing the FVIII gene behind an albumin promoter.\textsuperscript{40} These studies will help answer the question of whether such FVIII synthesis will reestablish a DDAVP-releasable FVIII pool. Furthermore, if the storage of FVIII is not achieved, the relative importance of the acute-phase increases in FVIII during normal hemostasis could be studied. Proteins expressed in the vascular endothelium will have immediate access to flowing blood through their apical surface. Whether synthesis in an adjacent cell (ie, hepatocytes) will be as efficient in the transport of active FVIII into the circulation awaits further study.

Our results demonstrate that nascent FVIII synthesis by transduced endothelial cells was functional and costored with vWF in all endothelial cell types studied. In our experimental model with the endothelial cell, post-Golgi segregation of FVIII into WPBs allowed it to be coreleased with vWF after agonist stimulation, thereby mimicking the observed physiological response in vivo.\textsuperscript{19} Although HUVECs do not respond to DDAVP,\textsuperscript{22,41} other agonists induce the release of functional FVIII from these storage sites. Recently, Kaufmann et al\textsuperscript{42} demonstrated that HUVECs can become responsive to DDAVP after transfection with the cDNA for the V2 receptor. This receptor is normally found in endothelial cells from other vascular sites.\textsuperscript{41} If some, or most, FVIII is produced in endothelium, endothelial cells that contain the endogenously active V2 receptor would be induced to release both FVIII and vWF after the administration of DDAVP. One such pool may exist in the hepatic sinusoidal endothelium (HSEC). Studies by Stel et al\textsuperscript{42} have detected FVIII antigen in liver sinususes, and more recently Do et al\textsuperscript{10} showed that cultured, isolated hepatic sinusoidal endotheliums synthesize and secrete FVIII.

Targeting of the vascular endothelium for the gene therapy of hemophilia A could result in the improved synthesis of FVIII and the establishment of a regulated storage pool of FVIII that could be subsequently released in times of stress or when pharmacologically induced by DDAVP. This would reestablish the activatable storage pool of both proteins. In regions of local vascular damage, local vWF and FVIII would be released and thereby provide improved local hemostatic effectiveness. Further studies are therefore indicated to determine the general extrapolation of our observations to normal vascular responses and the usefulness of endothelial cells as potential targets for gene therapy.

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References


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