BRCA1 is a novel target to improve endothelial dysfunction and retard atherosclerosis

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Objective: *BRCA1*, a tumor suppressor gene implicated in breast and ovarian cancers, exerts multiple effects on DNA repair and affords resistance against cellular stress responses. We hypothesized that *BRCA1* limits endothelial cell apoptosis and dysfunction, and via this mechanism attenuates atherosclerosis.

Methods: Loss and gain of function were achieved in cultured endothelial cells by silencing and overexpressing *BRCA1*, respectively. In vivo loss and gain of function were performed by generating endothelial cell-specific knockout (*EC-BRCA1*^{-/-}) mice and administering a *BRCA1* adenovirus. Well-established cell and animal models of angiogenesis and atherosclerosis were used.

Results: *BRCA1* is basally expressed in endothelial cells. *BRCA1* overexpression protected and *BRCA1* silencing exaggerated inflammation- and doxorubicin-induced endothelial cell apoptosis. Key indices of endothelial function were modulated in a manner consistent with an effect of *BRCA1* to limit endothelial cell apoptosis and improve endothelial function. *BRCA1* overexpression strongly attenuated the production of reactive oxygen species and upregulated endothelial nitric oxide synthase, phosphorylated endothelial nitric oxide synthase, phosphorylated Akt, and vascular endothelial growth factor- α expression. *BRCA1* overexpression also improved capillary density and promoted blood flow restoration in mice subjected to hind-limb ischemia. *BRCA1*-overexpressing *ApoE*^{-/-} mice fed a Western diet developed significantly less aortic plaque lesions, exhibited reduced macrophage infiltration, and generated less reactive oxygen species. Lung sections and aortic segments from *EC-BRCA1*^{-/-} mice demonstrated greater inflammation-associated apoptosis and impaired endothelial function, respectively. *BRCA1* expression was attenuated in the plaque region of human atherosclerotic carotid artery samples compared with the adjacent plaque-free area.

Conclusions: These data collectively highlight a previously unrecognized role of *BRCA1* as a gatekeeper of inflammation-induced endothelial cell function and a target to limit atherosclerosis. Translational studies evaluating endothelial function and atherosclerosis in individuals with *BRCA1* mutations are suggested. (J Thorac Cardiovasc Surg 2013;146:949-60)

Supplemental material is available online.

Germline mutations in the tumor suppressor *BRCA1* (BRCA1) are highly penetrant for the increased risk of breast and ovarian cancer.¹

Mutations in the *BRCA1* gene occur in up to 50% of inherited breast cancers, and individuals who carry a defective copy of the gene have a 50% to 85% cumulative lifetime risk of developing breast cancer and a 12% to 60% chance of developing ovarian cancer.¹ *BRCA1* functions as a caretaker of genome stability and exerts multiple effects on key cellular processes involved in DNA repair,

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Funding: This work was supported by grants from the Heart and Stroke Foundation of Canada and the Canadian Institutes of Health Research (to S.V.), an unrestricted Cardiology-Oncology Education Program grant from Sanofi-Aventis Canada Inc and Roche Canada (to C.B.-M. and S.V.), and an unrestricted grant from Abbott Canada. K.K.S. was the recipient of an Ontario Ministry of Research and Innovation's Post-Doctoral Fellowship and received the 2009 American Heart Association Vivien

Thomas Young Investigator Award for this work. P.C.S. was the recipient of a Fellowship from the Heart and Stroke/Richard Lewar Centre of Excellence in Cardiovascular Research. H.T. was the St Michael's Hospital Sanofi-Aventis Cardiometabolic Risk Initiative Research Fellow. W.L.S. is the Canada Research Chair in Integrative Stem Cell Biology at the University of Ottawa. S.V. is the Canada Research Chair in Atherosclerosis at the University of Toronto.

Disclosures: Authors have nothing to disclose with regard to commercial support. K.K.S. and P.C.S. share the position of first author.

Received for publication Oct 22, 2012; revisions received Dec 12, 2012; accepted for publication Dec 18, 2012; available ahead of print Feb 18, 2013.

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0022-5223/\$36.00

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<http://dx.doi.org/10.1016/j.jtcvs.2012.12.064>

Abbreviations and Acronyms

ad	= adenovirus
CMV	= cytomegalovirus
Dox	= doxorubicin
EC	= endothelial cell
ENOS	= endothelial nitric oxide synthase
FITC	= fluorescein isothiocyanate
GAPDH	= glyceraldehyde 3-phosphate dehydrogenase
GFP	= green fluorescent protein
HUVEC	= human umbilical vein endothelial cell
ICAM-1	= intercellular adhesion molecule 1
MOI	= multiplicity of infection
oxLDL	= oxidized low-density lipoprotein
PCR	= polymerase chain reaction
PFU	= plaque-forming unit
ROS	= reactive oxygen species
siRNA	= small interfering RNA
SNP	= single nucleotide polymorphism
TNF α	= tumor necrosis factor- α
VCAM-1	= vascular cell adhesion molecule 1
VEGF	= vascular endothelial growth factor

transcriptional regulation, ubiquitination, and cell-cycle control.²

DNA damage not only is important in cancer syndromes but also represents a common pathophysiologic basis for diverse diseases characterized by increased cellular and oxidative stress.^{3,4} Atherosclerosis, a leading cause of death worldwide, is a chronic inflammatory condition, and endothelial dysfunction plays an early and permissive role in the development and progression of the disease.⁵ A common mechanism through which cardiovascular risk factors lead to endothelial dysfunction is via increased generation of reactive oxygen species (ROS), thus elevating oxidative stress that in turn promotes DNA damage and endothelial cell (EC) apoptosis.⁴

We recently demonstrated in mice a cardioprotective and anti-inflammatory role for BRCA1.^{6,7} We have also reported on the cardioprotective nature of BRCA2 (Breast Cancer susceptibility gene 2).⁸ Because BRCA1 plays an integral role in promoting DNA repair, we hypothesized that BRCA1 also may be involved in regulating EC survival and function, and thus may retard the progression of atherosclerosis. We report for the first time that BRCA1 limits EC apoptosis, restores endothelial function, and attenuates atherosclerotic lesion development, thereby highlighting a novel role for BRCA1 as a regulator of vascular health. In addition to identifying a potentially new target for vascular diseases, these data may point toward a heightened susceptibility of *BRCA1* mutation carriers toward atherothrombotic vascular diseases.

MATERIALS AND METHODS

A detailed, expanded Materials and Methods section is available in the Online Supplement.

Cell Culture, Adenoviral Gene Delivery, and *BRCA1* Gene Silencing

Human umbilical vein endothelial cells (HUVECs) were cultured in MCDB-131 complete medium and used at passages 3 to 5. We used a custom-designed BRCA1 adenovirus (ad-BRCA1) that contained the complete coding sequence of human *BRCA1* (accession no. NM_007294.2) and was under the control of the cytomegalovirus (CMV) promoter. Where appropriate, ad-null (ad-CMV-null) or ad-green fluorescent protein (GFP) served as controls for ad-BRCA1. BRCA1 gene silencing was achieved with the small interfering RNA (siRNA) against human *BRCA1*, whereas a combination of 4 scrambled siRNAs served as the negative control.

Animal Studies

All animal-associated procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the St Michael's Hospital Animal Care Committee.

Male *BALB/c* mice from Charles River Laboratories International, Inc (Wilmington, Mass) were used for hind-limb ischemia studies.

ApoE^{-/-} mice were obtained from The Jackson Laboratory (Bar Harbor, Me) and divided into 3 study groups. The first and second groups were fed the Western diet for 4 and 16 weeks, respectively, while concurrently administered ad-GFP, ad-null, or ad-BRCA1. These mice were subsequently euthanized, and their aortas were isolated for immunohistochemical staining. The third group was fed the Western diet for 2 weeks, after which plasma samples were collected for lipid analyses.

EC-specific BRCA1 knockout mice were generated by crossing Tie2-Cre^{tg} mice (The Jackson Laboratory) with BRCA1^{fl/fl} mice (MMHCC, National Cancer Institute, Frederick, Md) to generate Tie2-Cre^{tg}; BRCA1^{fl/+} (*EC-BRCA1*^{+/-}) mice. *EC-BRCA1*^{+/-} mice were crossed with BRCA1^{fl/+} mice to generate Tie2-Cre^{tg} BRCA1^{fl/fl} (*EC-BRCA1*^{-/-}) mice, which demonstrate inactivation of the BRCA1 gene specifically in ECs.

Human Carotid Artery Samples

Study protocols were reviewed and approved by the Research Ethics Boards of St Michael's Hospital and Hamilton Health Sciences. Participation was voluntary, and all study subjects provided informed consent. Carotid artery segments were collected en bloc at the time of endarterectomy from the intima to the external elastic lamina. Two sections were taken from below ("normal") and at (plaque containing) the carotid bifurcation.

Apoptosis Assays

Apoptosis in HUVECs was induced with 20 ng/mL human recombinant tumor necrosis factor- α (TNF α), 10 μ g/mL oxidized low-density lipoprotein (oxLDL), or 2 μ mol/L doxorubicin (Dox). The extent of apoptosis was measured via flow cytometry by enumerating HUVECs positive for annexin V-fluorescein isothiocyanate (FITC) and propidium iodide. Total HUVEC genomic DNA samples were electrophoresed on 1% agarose gels and subsequently stained with ethidium bromide for assessment of DNA fragmentation. Lipopolysaccharide-induced lung injury as determined by the terminal deoxynucleotidyl transferase assay was examined in *EC-BRCA1*^{+/-} mice.

Western Blot Analyses, RNA Extraction, and Real-Time Polymerase Chain Reaction

Protein lysates were processed for Western blot analysis. Blots were probed for BRCA1, endothelial nitric oxide synthase (eNOS), Ser¹¹⁷⁷-phospho-eNOS, Akt, Ser⁴⁷³-phospho-Akt, cleaved caspase-3, vascular cell adhesion molecule 1 (VCAM)-1, intercellular adhesion molecule

1 (ICAM-1), E-selectin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Total RNA was prepared for polymerase chain reaction (PCR) or real-time PCR reactions. Transcript levels of *BRCA1*, VCAM-1, ICAM-1, E-selectin, vascular endothelial growth factor (VEGF)- α , and GAPDH were quantified.

Quantification of Reactive Oxygen Species and Vascular Endothelial Growth Factor Levels

ROS and VEGF α levels were determined with the OxiSelect ROS Assay Kit (Cell Biolabs, Inc, San Diego, Calif) and a VEGF ELISA Kit (Thermo Scientific, Burlington, Ontario, Canada) respectively.

In Vitro Angiogenesis and Migration

The angiogenic and migratory properties of HUVECs were examined with the In Vitro Angiogenesis Assay Kit (Chemicon, Temecula, Calif) and the CytoSelect 24-well Cell Migration Fluorometric Assay system (Cell Biolabs, Inc), respectively.

Immunohistochemical and Immunofluorescent Staining

Murine aortic root sections, human carotid artery sections, and en face aorta samples were subjected to routine immunohistochemical staining with BRCA1, Oil Red O, F4/80, or dihydroethidium. Sections of gastrocnemius muscles from the ischemic limbs of *BALB/c* mice were stained with rhodamine-conjugated isolectin-B4 and FITC-conjugated α -smooth muscle actin.

Vascular Function

Endothelial function of the aortic segments from *EC-BRCA1*^{-/-} mice was assessed via wire myography. Unilateral hind-limb ischemia was induced in 8-week-old male *BALB/c* mice. Limb perfusion was monitored by laser Doppler flow imaging for up to 28 days post-surgery.

Statistical Analysis

Unless otherwise stated, data are presented as mean $\pm$ standard deviation for the number of samples. The Student *t* test was applied when the means of 2 groups were being compared. Differences between multiple means were evaluated by analysis of variance, and when overall differences were detected, individual means were compared post hoc with the Bonferroni's test.

RESULTS

BRCA1 Is Basally Expressed in Human Umbilical Vein Endothelial Cells and Limits Human Umbilical Vein Endothelial Cell Apoptosis After Inflammatory and Genotoxic Stress

BRCA1 is basally expressed in HUVECs (Figure E1, A and B). To evaluate the functional relevance of BRCA1 in HUVECs, we used gain- and loss-of-function strategies. Optimal efficacy of adenoviral delivery was determined with ad-GFP to be 20 multiplicity of infection (MOI). GFP was detected 24 hours post-transfection in approximately 80% of HUVECs (Figure E1, C). Ad-BRCA1-mediated BRCA1 overexpression was confirmed by PCR and immunoblotting (Figure E1, A and B). The reader should note that although all of the subsequent studies were performed in 3 groups of HUVECs—naïve (nontransfected) HUVECs, ad-null-transfected HUVECs, and ad-BRCA1-transfected

HUVECs—only data from the ad-null- and ad-BRCA1-treated groups are shown because findings from the naïve and ad-null-transfected HUVECs were indistinguishable. TNF α evoked significant apoptosis in ad-null-transfected HUVECs but not in BRCA1-overexpressing HUVECs (Figure 1, A). This observation was confirmed via immunoblots for cleaved caspase-3 and DNA fragmentation assays, where BRCA1 overexpression dampened TNF α -associated increases in cleaved caspase-3 protein levels (Figure 1, B) and DNA fragmentation (Figure 1, C). That oxLDL not only elevated the percentage of apoptotic HUVECs (Figure 1, D) but also enhanced the levels of cleaved caspase-3 (Figure 1, E) was not unexpected because EC apoptosis induced by oxLDL is integral to the atherogenic pathway. Of more interest was the observation that oxLDL-triggered apoptosis also was significantly reduced in BRCA1-overexpressing HUVECs (Figure 1, D and E). Genotoxic stress caused by chemotherapeutic drugs, such as Dox, has been shown to activate apoptosis in ECs. We report marked apoptosis in Dox-treated ad-null-transfected HUVECs, an effect that was significantly reduced in ad-BRCA1-transfected HUVECs (Figure 1, F and G). Loss of function was performed with silenced-BRCA1 at an optimal concentration of 10 nM (Figure 1, H). Apoptosis in TNF α -treated *BRCA1*-silenced HUVEC cultures was significantly greater compared with that in TNF α -treated control HUVECs (Figure 1, I). These data suggest that BRCA1 protects HUVECs against apoptosis induced by inflammatory and genotoxic stresses.

To gain critical insights into the role of BRCA1 in endothelial regulation and to circumvent the embryonic lethality associated with systemic BRCA1 loss,⁹ we generated *EC-BRCA1*^{+/-} and *EC-BRCA1*^{-/-} mice. These mice were viable, fertile, and born in the expected Mendelian ratios (data not shown). Terminal deoxynucleotidyl transferase staining revealed that, in comparison with the wild-type littermate control group, lung sections from *EC-BRCA1*^{+/-} mice exhibited more extensive lipopolysaccharide-associated endothelial apoptosis (Figure 1, J).

BRCA1 Impairs Tumor Necrosis Factor- α -Induced Reactive Oxygen Species Generation in Human Umbilical Vein Endothelial Cells

BRCA1 reduces the generation of ROS,^{10,11} a critical mediator of EC apoptosis elicited by TNF α ,¹² oxLDL,¹³ and Dox.¹⁴ We observed significantly lower levels of ROS in ad-BRCA1-transfected HUVECs compared with ad-null-treated HUVECs after TNF α stimulation (Figure 1, K). Furthermore, we detected significantly higher levels of ROS in TNF α -treated BRCA1-silenced HUVECs relative to HUVECs that had been treated with the control siRNA (Figure 1, L). These data demonstrate the importance of BRCA1 in controlling ROS generation in HUVECs and arguably HUVEC apoptosis.

FIGURE 1. BRCA1 limits HUVEC apoptosis and ROS generation after inflammatory and genotoxic stress. A-C, After transfection with ad-null or ad-BRCA1, HUVECs were incubated with 20 ng/mL TNF α or its diluent. A, Representative flow cytometry dot plots are shown on the left with the percentage of total apoptotic cells (quadrants S2 + S4) depicted quantitatively on the right. $n = 3$ in triplicate. * $P < .01$ versus ad-null group. † $P < .01$ versus ad-null + TNF α group. B, Representative immunoblots for cleaved caspase-3 and GAPDH. $n = 3$ in triplicate. C, Total genomic DNA underwent agarose gel electrophoresis for visualization of DNA fragmentation. The marker was from a nonadjacent lane on the same gel. D and E, After transfection, HUVECs were treated with 10 μ g/mL oxLDL or its diluent. D, Apoptosis was quantified as described in A. $n = 3$ in triplicate. * $P < .01$ versus ad-null group. † $P < .01$ versus ad-null + oxLDL group. E, Representative immunoblots for cleaved caspase-3 and GAPDH. $n = 3$ in triplicate. F and G, After transfection, HUVECs were treated with 10 μ g/mL Doxorubicin or its diluent. F, Apoptosis was quantified as described in A. $n = 3$ in triplicate. * $P < .01$ versus ad-null group. † $P < .01$ versus ad-null + Doxorubicin group. G, Representative immunoblots for cleaved caspase-3 and GAPDH. $n = 3$ in triplicate. H, Representative immunoblots for BRCA1 and GAPDH. $n = 3$ in triplicate. I, Apoptosis was quantified as described in A. $n = 3$ in triplicate. * $P < .01$ versus Control siRNA group. † $P < .01$ versus Control siRNA + TNF α group. J, Representative TUNEL assay images and bar graph showing % TUNEL-positive Nuclei for WT and EC-BRCA1^{-/-} cells. $n = 3$ in triplicate. * $P < .01$ versus WT group. K, Representative bar graph showing DCF Fluorescence (AU) for ad-null, ad-BRCA1, ad-null+TNF α , and ad-BRCA1+TNF α . $n = 3$ in triplicate. * $P < .01$ versus ad-null group. † $P < .01$ versus ad-null + TNF α group. L, Representative bar graph showing DCF Fluorescence (AU) for Control siRNA, siBRCA1, Control siRNA+TNF α , and siBRCA1+TNF α . $n = 3$ in triplicate. * $P < .01$ versus Control siRNA group. † $P < .01$ versus Control siRNA + TNF α group.

BRCA1 Restores Key Indices of Endothelial Function

TNF α significantly impaired the capacity of HUVECs to form capillary-like tubular structures, an effect that was not evident in BRCA1-overexpressing HUVECs (Figure 2, A). The marked attenuation of HUVEC migratory capacity by TNF α was restored in BRCA1-overexpressing HUVECs (Figure 2, B). VCAM-1, ICAM-1, and E-selectin have fundamental roles in maintaining endothelial function, and disruptions in their normal expression or function can culminate in endothelial dysfunction. BRCA1 overexpression in HUVECs decreases VCAM-1 expression.¹⁰ In our hands, transcript and protein levels of VCAM-1, ICAM-1, and E-selectin were significantly upregulated by TNF α in ad-null-treated HUVECs; these increases were appreciably attenuated in BRCA1-overexpressing HUVECs (Figure 2, C-E). These findings suggest a pivotal role for BRCA1 in limiting inflammation-induced HUVEC activation and in restoring the angiogenic capacity of HUVECs. Aortic rings from *EC-BRCA1*^{-/-} mice were significantly less responsive to acetylcholine (Figure 2, F), suggestive of impaired endothelial function ($E_{\max}$ for rings from *EC-BRCA1*^{-/-} mice = 58.23% $\pm$ 8.00%; $E_{\max}$ for rings from wild-type mice = 81.38% $\pm$ 6.70%) providing cogent ex vivo evidence that BRCA1 is essential to endothelial homeostasis. Smooth muscle function was unaffected by loss of the *BRCA1* gene as evidenced by the similar phenylephrine- and sodium nitroprusside-induced responses recorded in aortic rings from *EC-BRCA1*^{-/-} mice and their littermate controls (data not shown).

BRCA1 Promotes Endothelial Nitric Oxide Synthase, Phosphorylated Endothelial Nitric Oxide Synthase, Phosphorylated Akt, and Vascular Endothelial Growth Factor- α Expression

ECs constitutively express eNOS, a critical regulator of endothelial function that has been reported to be downregulated by TNF α . We observed that TNF α markedly reduced eNOS levels in ad-null-transfected HUVECs and that

eNOS levels were significantly upregulated in BRCA1-overexpressing TNF α -treated HUVECs (Figure 3, A). eNOS is a dynamic enzyme that is regulated in part by Akt-dependent phosphorylation at the Ser¹¹⁷⁷ residue.¹⁵ TNF α -stimulated BRCA1-overexpressing HUVECs exhibited increased eNOS-phosphorylation at the Ser¹¹⁷⁷ residue (Figure 3, A) and significantly greater Akt phosphorylation (Figure 3, B). BRCA1 overexpression enhances VEGF α expression,¹⁶ and *BRCA1* mutations in humans are associated with significantly reduced serum VEGF α levels.¹⁷ We report an approximately 15-fold upregulation of VEGF α in BRCA1-overexpressing, TNF α -treated HUVECs relative to that in ad-null-transfected, TNF α -treated HUVECs (Figure 3, C). VEGF α secretion in ad-BRCA1-transfected, TNF α -treated HUVECs was significantly higher than that in the corresponding ad-null-treated group (Figure 3, D). These findings indicate that BRCA1 stimulates eNOS expression at least in part via upregulation of VEGF α and triggers eNOS activation through Akt phosphorylation.

BRCA1 Gene Therapy Improves Blood Flow Recovery in Ischemic Hind Limbs

To evaluate the potential translational implications of improved endothelial function in vivo, we examined the extent of neovascularization that occurred after induction of hind-limb ischemia in *BALB/c* mice. BRCA1 overexpression was confirmed through immunoblotting of tissue lysates isolated from adductor muscles 8 days post-surgery (Figure 4, A). As early as 8 days post-surgery, the ischemic limbs of ad-BRCA1-treated mice demonstrated significantly greater perfusion than those of mice that had been administered ad-null (Figure 4, B and C). Perfusion in the ischemic limb was consistently and significantly greater in ad-BRCA1-treated mice (Figure 4, B and C). Enhanced blood flow recovery in mice treated with ad-BRCA1 versus ad-null was associated with higher capillary densities at 28 days post-ischemia induction

versus ad-null + oxLDL group. E, Representative immunoblots for cleaved caspase-3 and GAPDH. n = 3 in triplicate. F and G, After transfection, HUVECs were incubated with 2 μ mol/L Dox or its diluent. F, Apoptosis was quantified as described in A. n = 3 in triplicate. * P < .01 versus ad-null group. † P < .01 versus ad-null + Dox group. G, Representative immunoblots for cleaved caspase-3 and GAPDH. n = 3 in triplicate. H, Representative BRCA1 and GAPDH immunoblots derived from the total protein lysates of HUVECs transfected with the control (scrambled) siRNA or siBRCA1 before exposure to TNF α . n = 3 in triplicate. Solid lines through immunoblots demarcate nonadjacent lanes from the same Western blot. I, After treatment with siBRCA1 or the control siRNA, HUVECs were incubated with 20 ng/mL TNF α before the apoptotic cells were enumerated. n = 3 in triplicate. * P < .01 versus control siRNA group. † P < .01 versus control siRNA + TNF α group. J, Representative micrographs and quantification of terminal deoxynucleotidyl transferase-positive nuclei (arrows) in lung sections from lipopolysaccharide-treated *EC-BRCA1*^{+/-} and wild-type littermate controls. n = 3. * P < .05 versus control. K, After transfection, HUVECs were incubated with 20 ng/mL TNF α or its diluent before ROS levels were determined by measuring 2', 7'-dichlorodihydrofluorescein fluorescence. n = 12. * P < .05 versus ad-null group. † P < .05 versus ad-null + TNF α group. L, ROS production was measured in HUVECs treated with control (scrambled) siRNA or siBRCA1 before incubation with TNF α . n = 12. * P < .05 versus scrambled siRNA group. † P < .05 versus scrambled siBRCA1 + TNF α group. *BRCA1*, BRCA1, BRCA1 susceptibility gene 1; *ad*, adenovirus; *ad-BRCA1*, BRCA1 adenovirus; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *TNF α* , tumor necrosis factor- α ; *EC*, endothelial cell; *ROS*, reactive oxygen species; *siRNA*, small interfering RNA; *HUVEC*, human umbilical vein endothelial cell; *mRNA*, messenger RNA; *oxLDL*, oxidized low-density lipoprotein.

FIGURE 2. BRCA1 restores key indices of endothelial function after TNF α treatment. A, After transfection with ad-null or ad-BRCA1, HUVECs were transferred to a Matrigel-coated plate (BD Biosciences, San Jose, Calif) and treated with TNF α or its diluent. Representative micrographs showing capillary-like tube formation in HUVECs 5 hours after treatment with TNF α . Tubular structures in 4 fields per group were semiquantitatively analyzed. $n = 4$ to 5 in triplicate. * $P < .01$ versus ad-null group. † $P < .01$ versus ad-null + TNF α group. B, After transfection, HUVECs were treated with TNF α or its diluent before the extent of cell migration was evaluated. $n = 4$. * $P < .01$ versus ad-null group. † $P < .01$ versus ad-null + TNF α group. C-E, Total RNA and protein were extracted from ad-null and ad-BRCA1-transfected HUVECs that were subsequently treated with TNF α or its diluent. VCAM-1 (C), ICAM-1 (D), and E-selectin (E) transcript and protein levels after TNF α treatment. $n = 3$. * $P < .01$ versus ad-null group. † $P < .01$ versus ad-null + TNF α group. F, Phenylephrine-contracted (10^{-6} mol/L) thoracic aorta segments from wild-type and EC-BRCA1^{-/-} mice were challenged with 10^{-6} mol/L acetylcholine to assess endothelial function. $n = 4$ to 5. * $P < .05$ versus wild-type control. BRCA1, BRCA1, Breast Cancer susceptibility gene 1; ad, adenovirus; ad-BRCA1, BRCA1 adenovirus; EC, endothelial cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TNF α , tumor necrosis factor- α ; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; WT, wild type; HUVEC, human umbilical vein endothelial cell; mRNA, messenger RNA.

(Figure 4, D). These data suggest an important angiogenic role for BRCA1 in vivo.

BRCA1 Limits Atherosclerotic Lesion Formation

We confirmed successful delivery of ad-GFP by imaging the extent of GFP fluorescence in the aortic roots of *ApoE*^{-/-} mice 4 weeks after initiation of the diet and adenoviral treatment regimen (Figure E2, A). Immunohistochemical staining of aortic root sections and immunoblots performed with whole aorta lysates also revealed higher levels of BRCA1 in ad-BRCA1-treated *ApoE*^{-/-} mice relative to that in *ApoE*^{-/-} mice administered ad-null (Figure E2, B and C). Because the adenoviral vector used in the current study contained the CMV promoter, the expression of BRCA1 as a result of treatment with the

ad-BRCA1 was not specific to the endothelium and was also detected in cells other than aortic ECs (Figure E2, B). Accordingly, we cannot exclude the involvement of cell types other than ECs in the antiatherosclerotic effects described in the subsequent figures.

Oil Red O staining suggest that mice treated with ad-BRCA1 developed significantly less plaque compared with those that had received ad-null (Figure 5, A). Immunohistochemical staining demonstrated less macrophage infiltration into the aortic roots, which is indicative of less inflammation in ad-BRCA1-treated mice, compared with ad-null-treated mice, maintained on the Western diet for 4 weeks (Figure 5, B). The less-extensive plaque deposition and less severe inflammatory response that were associated with ad-BRCA1 treatment occurred in parallel with

FIGURE 3. BRCA1 enhances eNOS, phosphorylated eNOS, phosphorylated Akt, and VEGFa expression. After transfection with ad-null or ad-BRCA1, HUVECs were incubated with 20 ng/mL TNFα or its diluent. Representative immunoblots and semiquantitative data for (A) eNOS, phosphorylated eNOS, and GAPDH, and (B) Akt, phosphorylated Akt, and GAPDH. C, Real-time PCR analysis of VEGFa transcript levels. D, VEGFa levels as determined by enzyme-linked immunosorbent assay. n = 12. **P* < .01 versus ad-null group. †*P* < .01 versus ad-null + TNFα group. *BRCA1*, BRCA1 adenovirus; *ad*, Adenovirus; *ad-BRCA1*, BRCA1 adenovirus; *eNOS*, endothelial nitric oxide synthase; *p-eNOS*, phosphorylated endothelial nitric oxide synthase; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *TNFα*, tumor necrosis factor-α; *VEGF*, vascular endothelial growth factor; *mRNA*, messenger RNA.

lower ROS production (Figure 5, C). Plaque deposition in ad-BRCA1-treated *ApoE*^{-/-} mice (vs ad-null-treated *ApoE*^{-/-} mice) was continuously and significantly lower up to week 16 of the diet-adenoviral treatment (Figure 5, D). The liver is often the main target for injected ad vectors. Considering the role of the liver in the regulation of lipid

metabolism and the implications of dysfunctional lipid biology in the development and progression of atherosclerosis, we collected plasma for lipid measurements from *ApoE*^{-/-} mice that had been on the Western diet for 2 weeks. Lipid profiles of these mice were unaffected by the diet-adenoviral treatment strategy (Table E1).

FIGURE 4. BRCA1 improves recovery of ischemic hind limb. A, Representative immunoblot showing endogenous expression and BRCA1 overexpression in murine adductor muscles 8 days post-surgery. $n = 2/\text{group}$. B, Temporal changes in hind-limb perfusion. Data were calculated as the recovery of blood flow to the ischemic foot normalized to the contralateral nonischemic foot. $n = 6$ to 8. * $P < .05$ versus ad-null-treated group. C, Laser Doppler perfusion images of adenoviral-treated ischemic and nonischemic contralateral hind-limbs at day 0 and day 8 post-surgery. D, Gastrocnemius muscles were harvested 28 days post-surgery for histologic processing. Micrographs depict capillary density in sections of gastrocnemius muscle as discerned by rhodamine-conjugated isolectin-B4-positive areas (red, arrowheads). Arterioles were discriminated with FITC-conjugated smooth muscle α -actin (green, arrows). $n = 6$ -8. * $P < .05$ versus ad-null-treated group. *BRCA1*, BRCA1 susceptibility gene 1; *ad-BRCA1*, BRCA1 adenovirus; *ad*, adenovirus; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *FITC*, fluorescein isothiocyanate.

Reduced Expression of BRCA1 in Human Carotid Artery Plaques

To gather insight into the potential involvement of BRCA1 in the atherogenic process in humans, we measured BRCA1 expression and levels in human carotid artery samples harvested from the point of bifurcation and the adjacent control plaque-free area below the bifurcation (Figure 5, E). Atherosclerosis in carotid arteries tends to occur stereotypically in the carotid bulb at or above the bifurcation, whereas the region proximal to the bifurcation appears to be grossly uninvolved in this pathology.¹⁸ BRCA1 mRNA and protein levels in plaque-containing segments were significantly lower compared with those in the control

segments (Figure 5, F and G). Immunohistochemistry confirmed that segments obtained from the atherosclerotic plaque region had less BRCA1-positive areas than those from the control regions below the bifurcation point (Figure 5, H).

DISCUSSION

Atherosclerosis, a leading cause of death and disability worldwide, is a chronic inflammatory condition with EC apoptosis and endothelial dysfunction playing early and permissive roles in the development, progression, and clinical course of the disease.⁵ The strategic location of the endothelium allows it to “sense” changes in

FIGURE 5. BRCA1 limits atherosclerosis lesion formation in vivo, and BRCA1 expression is reduced in human carotid artery plaques. *ApoE*^{-/-} mice were treated with ad-GFP, ad-null, or ad-BRCA1 every 2 weeks while being maintained on the Western diet. A, Representative micrograph and quantification of Oil Red O–defined atherosclerotic lesions (red orange) in the aortic roots of ad-null or ad-BRCA1-injected *ApoE*^{-/-} mice fed the Western diet for 4 weeks. n = 9/group. **P* < .01 versus ad-null–treated group. B, Representative micrograph and quantification of macrophage sequestration, as defined by F4/80 staining (brown), in the aortic roots of ad-null or ad-BRCA1-injected *ApoE*^{-/-} mice fed the Western diet for 4 weeks. n = 9/group. **P* < .01 versus ad-null–treated group. C, Representative micrograph and quantification of dihydroethidium staining (red, ROS production) in aortic root sections from *ApoE*^{-/-} mice maintained on the Western diet for 4 weeks. n = 9/group. **P* < .01 versus ad-null–treated group. Data are presented as the mean of 4 to 5 fields as positive stained percent of total root area. D, Representative samples and quantification of Oil Red O–defined atherosclerotic lesions in en face preparations of descending aortas from *ApoE*^{-/-} mice maintained on the Western diet for 16 weeks and administered ad-null or ad-BRCA1 every second week. n = 9/group. **P* < .01 versus ad-null–treated group. E, Diagram outlines the control and plaque containing regions. Total RNA and protein were extracted from carotid artery samples for determination of BRCA1 levels via (F) real-time PCR analysis, and (G) immunoblotting. n = 3 to 4. **P* < .05 versus control. H, Representative micrographs showing BRCA1 positivity (brown) in carotid artery sections obtained from control and plaque-containing regions. n = 3 to 4. BRCA1, BRCA1 susceptibility gene 1; ad-BRCA1, BRCA1 adenovirus; ad, adenovirus; DHE, dihydroethidium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction; ROS, reactive oxygen species; GFP, green fluorescent protein.

hemodynamic forces and blood-borne signals, and “respond” by releasing a number of bioactive factors. EC apoptosis and dysfunction disrupt the balance between these bioactive factors, thereby predisposing the vessel wall to vascular inflammation, leukocyte adherence, platelet activation, pro-oxidation, thrombosis, and impaired coagulation leading to atherosclerosis.^{3,4}

DNA damage–induced apoptosis is purported to play an important role in the development of endothelial

dysfunction and atherosclerosis. This line of thought is strengthened by recent data showing that deficiency in the ataxia telangiectasia mutated kinase, a critical regulator of DNA damage repair, worsens features of metabolic syndrome and accelerates atherosclerosis in *ApoE*^{-/-} mice.¹⁹ It has also been reported that atherosclerosis is decreased in *ApoE*^{-/-} mice treated with an ataxia telangiectasia mutated kinase activator that is suggestive of a causal role for DNA damage-response pathways in atherosclerosis.¹⁹

Although the role of BRCA1 in DNA damage repair responses has been largely studied in cancer, this work supports the contention that BRCA1 is a critical modulator of EC apoptosis, endothelial dysfunction, and atherosclerosis, all of which are characterized by aberrant DNA damage and cellular stress. We provide evidence that BRCA1 is expressed in HUVECs and that it is capable of protecting HUVECs from undergoing apoptosis induced by various stressors that include TNF α , oxLDL, and Dox. We also demonstrate that *BRCA1* silencing exaggerates TNF α -induced HUVEC apoptosis.

Both inflammation and genotoxic stress induce apoptosis via ROS generation,¹²⁻¹⁴ and ROS-induced DNA damage may play an important role in the development of cancer and inflammatory diseases. Evidence to date suggests that several proatherogenic stimuli trigger ROS production in ECs and that the resultant oxidative stress plays a key role in mediating the pathologic manifestations of EC apoptosis and endothelial dysfunction that are associated with atherosclerosis. Because BRCA1 not only affords protection against DNA damage but also reduces ROS generation,^{10,11} we measured ROS levels in HUVECs after TNF α treatment and found that BRCA1 overexpression reduces and *BRCA1* silencing enhances TNF α -induced ROS production, thereby confirming that BRCA1 may protect ECs against apoptosis by negatively regulating stress-induced ROS generation.

In the milieu of cardiovascular risk factors that disturb vascular homeostasis, the endothelium becomes dysfunctional resulting in perturbed angiogenesis and enhanced expression of cellular adhesion molecules by the endothelium. Our current data indicate that key indices of endothelial function, such as the ability of ECs to form capillary-like tubes, migrate, and express surface markers of endothelial injury (VCAM-1, ICAM-1, and E-selectin), and the reduced response of aortic rings from *EC-BRCA1*^{-/-} mice to acetylcholine ex vivo, are modulated in a manner consistent with an effect of BRCA1 to limit EC apoptosis and improve endothelial function. Indeed, our results demonstrating significant upregulation of eNOS and VEGFa expression, as well as elevated Akt activation in BRCA1-overexpressing TNF α -stimulated HUVECs, suggest that the restorative nature of BRCA1 on endothelial function occurs at least in part through enhanced eNOS expression via VEGFa upregulation and eNOS activation by means of Akt phosphorylation, both of which are recognized survival mechanisms in ECs.

There seems to be no consensus as yet on the associative relationship between BRCA1 and VEGFa expression and activity. Kawai and colleagues²⁰ demonstrated that wild-type BRCA1 inhibited VEGFa promoter activity and secretion in breast cancer cells by modulating the estrogen receptor in a dose-dependent manner and primarily in the presence of estrogen. In contrast to the study by Kawai and colleagues but in agreement with ours, Kang and

colleagues¹⁶ found that elevation of BRCA1 levels in breast cancer cells enhanced VEGF promoter activity after exposure to conditions of hypoxia and increased VEGF mRNA levels. The same authors showed that silencing of endogenous BRCA1 resulted in decreased hypoxia-stimulated VEGF promoter activity and secretion. In an article by Welch and King,²¹ overexpression of wild-type BRCA1 in prostate and breast cancer cell lines was associated with an approximately 2.5-fold increase in VEGFa expression in the setting of oxidative stress.²¹ At the clinical level, significantly lower levels of VEGF were detected in patients with mutation of the BRCA1 gene when compared with patients with breast cancer without this mutation.¹¹ The balance of current data suggests that how BRCA1 modulates VEGFa expression and activity may be particularly dependent on the cell type, the presence of necessary cofactors, and whether the cells being studied are primary cells or a transformed cancer cell line.

By using a mouse model of neoangiogenesis after induction of hind-limb ischemia, we successfully translated our in vitro data to the in vivo level. Treatment with ad-BRCA1 not only improved capillary density but also restored blood flow to the ischemic limb. *BRCA1* gene therapy also limited the formation of atherosclerotic lesions and attenuated inflammation, macrophage infiltration, and ROS generation but had no effect on lipid metabolism. These data are critical because they represent an accepted translational end point of progressive endothelial dysfunction. Of note, the benefits of BRCA1 on endothelial dysfunction and ROS may be more applicable to the chronic experiments on endothelial dysfunction and atherosclerosis, as opposed to the setting of acute reperfusion injury, which shares many of the contributory factors. We also provide preliminary clinical confirmation in human atherosclerotic plaques wherein reduced BRCA1 levels are observed relative to plaque-free areas of the same vessel.

Our work collectively highlights a previously unrecognized role of BRCA1 as a gatekeeper of EC survival and function, and through this mechanism a novel target to improve ischemic neovascularization and attenuate atherosclerosis. Our data may also provide clues regarding the potential susceptibility of patients with mutations of the *BRCA1* gene to atherosclerotic vascular disease, in addition to cancer. Reports have suggested unknown effects of BRCA1 mutations on non-neoplastic deaths, particularly at older ages.²² Individuals with *BRCA1* mutations seem to face a 2-fold increased risk of developing diabetes, a disease characterized by increased endothelial dysfunction, inflammation, and atherosclerosis.²³ Single nucleotide polymorphisms (SNPs) in the *BRCA1*-associated protein have been linked to risk of myocardial infarction in Asian populations.²⁴ Zbuk and colleagues²⁵ recently reported an association between 2 BRCA2 SNPs and cardiovascular disease in a multiethnic population,²⁵ and Asselbergs and

colleagues²⁶ found a strong association between 2 other BRCA2 SNPs and low-density lipoprotein and total cholesterol levels in a large-scale, gene-centric meta-analysis. Collectively, these studies support the contention for clinical and population-based research.

Ad- or lentivirus-mediated tissue-specific (myocardial) and systemic gene therapy have successfully been demonstrated.^{7,27-29} Our data suggest that BRCA1 cell or gene therapy approaches that target DNA damage repair mechanisms may represent a novel approach to treat native and vein graft atherosclerosis—diseases characterized by endothelial dysfunction. This therapy may have additional benefits in surgical patients who develop severe sepsis, a disease also characterized by pronounced endothelial injury. Indeed, we have recently demonstrated that BRCA1 gene delivery before induction of experimental sepsis reduces systemic inflammatory response and multiple organ failure and improves survival in mice.⁷ The benefits of BRCA1 on cardiovascular protection seem to involve both the vascular endothelium and the myocardium. Our previous work⁶ coupled with the current article suggests that BRCA1-based gene therapy may have the potential to limit both ischemic and genotoxic cardiac failure, while also limiting the process of endothelial injury and atherogenicity.

Study Limitations

This study has a few limitations that should be mentioned. Because the ad vectors that we used were under the control of the CMV promoter, the results that we report cannot be specifically associated with ECs. Accordingly, we are unable to rule out any role that may be played by other cell types as a result of the ensuing BRCA1 overexpression. Although our data from the human carotid samples suggest a potential association between reduced BRCA1 expression and plaque formation, they are preliminary and speculative, and do not provide any evidence of causality.

CONCLUSIONS

Several lines of investigation are warranted. Definitive studies evaluating plaque burden in EC-specific BRCA1 knockout on the apolipoprotein-E background (EC-BRCA1^{-/-}; ApoE^{-/-} double knockout) mice should be performed. Studies in larger animal models of coronary endothelial dysfunction (eg, the porcine model) also should be performed to confirm these observations. Finally, genetic studies evaluating whether SNPs in BRCA1/2 are linked to cardiovascular disease, such as those by Zbuk and colleagues²⁵ and Asselbergs and colleagues,²⁶ should be performed.

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EXPANDED MATERIALS AND METHODS

Cell Culture, Adenoviral Gene Delivery, and *BRCA1* Gene Silencing

HUVECs and MCDB-131 complete medium were obtained from Clonetics (VEC Technologies, Inc, Rensselaer, NY). Studies were performed on cultured HUVECs at passages 3 to 5.

We used a custom-designed *BRCA1* adenovirus (ad-*BRCA1*; Vector BioLabs, Philadelphia, Pa) that contained the complete coding sequence of human *BRCA1* (accession no. NM_007294.2) and was under the control of the CMV promoter. Ad-null (ad-CMV-null) or ad-GFP (both from Vector BioLabs), both also under the control of the CMV promoter, served as controls for ad-*BRCA1*. The entire transfection process took 24 hours, after which HUVECs were processed as required for various analyses. As shown in Figure E1, optimal adenoviral transfection efficacy was achieved at 20 MOI.

Before study initiation, the siRNA against human *BRCA1* (*BRCA1* siRNA SMARTpool) or the negative control (consisting of 4 scrambled siRNAs) were incubated with OptiMEM (Invitrogen, Burlington, Ontario, Canada) and the siPORT NeoFX Transfection Agent (Ambion, Burlington, Ontario, Canada) per the manufacturer's instructions. HUVECs were plated in 6-well plates at a density of 1.5×10^5 /well for the 24-hour transfection process. Optimal si*BRCA1* concentration was determined in preliminary studies to be 10 nM. After transfection, HUVECs were washed in PBS and maintained for an additional 24 hours in MCDB-131 complete medium before incubation with TNF α for 24 hours. HUVECs were then processed for flow cytometry studies or protein expression analysis.

Animal Studies

All of the procedures that were performed on animals were in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the St Michael's Hospital Animal Care Committee.

Male *BALB/c* mice were from Charles River Laboratories International, Inc (Wilmington, Mass), and *ApoE*^{-/-} mice were from The Jackson Laboratory (Bar Harbor, Me).

EC-specific *BRCA1* knockout mice were generated by crossing Tie2-Cre^{tg} mice (The Jackson Laboratory), wherein the Tie2 promoter drives Cre recombinase expression, with *BRCA1*^{fl/fl} (where exon 11 of the *BRCA1* gene is flanked by 2 loxP sites) mice (MMHCC, National Cancer Institute, Frederick, Md) to generate Tie2-Cre^{tg}; *BRCA1*^{fl/+} (*EC-BRCA1*^{+/-}) mice. *EC-BRCA1*^{+/-} mice were crossed with *BRCA1*^{fl/+} mice to generate Tie2-Cre^{tg}; *BRCA1*^{fl/fl} (*EC-BRCA1*^{-/-}) mice, which demonstrate inactivation of the *BRCA1* gene specifically in ECs.

Human Carotid Artery Samples

Study protocols were reviewed and approved by the Research Ethics Boards of St Michael's Hospital and Hamilton Health Sciences before study commencement. Participation was voluntary, and all study subjects provided informed consent. Carotid artery segments were collected en bloc at the time of endarterectomy from the intima to the external elastic lamina. Two sections were taken from below ("normal") and at (plaque containing) the carotid bifurcation. One was snap-frozen in liquid nitrogen, and the other was fixed and embedded in Tissue-Tek optimum cutting temperature (Sakura Finetek, Torrance, Calif).

Apoptosis Assays

Human recombinant TNF α (R&D, Burlington, Ontario, Canada), oxLDL (Biomedical Technologies Inc, Stoughton, Mass), and Dox (Sigma, Oakville, Ontario, Canada) were purchased. Optimal TNF α , oxLDL, and Dox concentrations for inducing significant apoptosis after 24 hours of treatment were determined in preliminary experiments to be 20 ng/mL, 10 μ g/mL, and 2 μ mol/L, respectively. Where appropriate, the control

groups were treated with the respective diluents: PBS for TNF α and oxLDL, and water for Dox.

HUVECs, at 50% to 60% confluency, were transfected with ad-null or ad-*BRCA1*. At the end of the transfection process, HUVECs were treated with TNF α , Dox, or oxLDL for an additional 24 hours before the extent of apoptosis was measured by enumerating HUVECs positive for Annexin V-FITC and propidium iodide (both from BD Biosciences, Mississauga, Ontario, Canada) via flow cytometry. Fluoresceins were excited at 488 nm, and emissions were detected at 512 nm and 543 nm on a Cytomics FC500 system (Beckman Coulter Inc, Carlsbad, Calif). The dots in quadrants S2 and S4 represent late and early apoptotic events, respectively. The percentage of total apoptotic cells (S2 + S4) is presented in the accompanying graphs as percent apoptotic cells.

HUVECs were cultured in 100-mm dishes and transfected with ad-null or ad-*BRCA1* on reaching 50% to 60% confluency. At the end of the transfection protocol, HUVECs were treated with TNF α or its diluent for an additional 24 hours before total genomic DNA was extracted using standard protocols and 20 μ g of genomic DNA electrophoresed on 1% agarose gels that were subsequently stained with ethidium bromide for assessment of DNA fragmentation.

Lipopolysaccharide-induced lung injury as determined by the terminal deoxynucleotidyl transferase assay was examined in *EC-BRCA1*^{+/-} mice.

Western Blot Analyses

Protein lysates from HUVECs, mouse, and human tissues were extracted in RIPA buffer (Sigma) supplemented with a protease inhibitor cocktail (Roche, Mississauga, Ontario, Canada). Protein concentrations were determined with the Bradford Dye Binding Assay (Bio-Rad, Mississauga, Ontario, Canada), and 40- to 80- μ g sample loads were resolved on sodium dodecyl sulfate-polyacrylamide gels, electrotransferred onto nitrocellulose membranes, and probed with antibodies against *BRCA1* (1:200), eNOS (1:1000), Ser¹¹⁷-phospho-eNOS (1:1000), Akt (1:1000), Ser⁴⁷³-phospho-Akt (1:1000), cleaved caspase-3 (1:1000), VCAM-1 (1:1000), ICAM-1 (1:1000), E-selectin (1:1000), or GAPDH (1:2000). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies, immunocomplexes were visualized by enhanced chemiluminescence (GE Healthcare, Mississauga, Ontario, Canada), and band intensity was quantified by densitometry using ImageJ software. *BRCA1* and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif). The GAPDH antibody was from Chemicon (Ontario, Canada). The eNOS antibody was from BD Biosciences (Temecula, Calif). VCAM-1 and E-selectin antibodies were from Abcam (Cambridge, Mass). All other antibodies were from Cell Signaling Technology, Inc (Danvers, Mass).

RNA Extraction and Real-Time Polymerase Chain Reaction

Total RNA were extracted with the RNeasy Plus Mini Kit (Qiagen, Toronto, Ontario, Canada) and reverse-transcribed with the Quantitect Reverse Transcription Kit (Qiagen) before undergoing PCR or real-time PCR on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, Calif). Transcripts were quantified using the following primer pairs: 5'-TCAAGGAACCTGTCTCCACA-3' and 5'-CTTCTTGTAGGCTCCTTTTGG-3' for *BRCA1*, 5'-TGGGAAAAACAGAAAAGAGGTG-3' and 5'-GTCTCCAATCTGAGCAGCAA-3' for VCAM-1, 5'-TGATGGGCAGTCAACAGCTA-3' and 5'-AGGGTAAGGTTCTTGCCAC-3' for ICAM-1, 5'-AAGCCTTGAATCAGACGGAA-3' and 5'-TCCCTCTAGTCCCCAGATG-3' for E-selectin, 5'-CTACCTCCACCATGCCAAGT-3' and 5'-AGCTGCGCTGATAGACATCC-3' for VEGFa, and 5'-CACCAGGCTGCTTTAACTCTGGTA-3' and 5'-CCTTGA CGGTGCCATGGAATTTGC-3' for GAPDH.

Quantification of Reactive Oxygen Species and Vascular Endothelial Growth Factor Levels

HUVECs underwent transfection or silencing before they were treated for 12 hours with TNF α . ROS production was determined by the fluorescent intensity of 2', 7'-dichlorodihydrofluorescein (ex/em: 480 nm/530 nm) using the OxiSelect ROS Assay Kit (Cell Biolabs, Inc, San Diego, Calif). VEGF α levels in HUVECs that had been transfected with ad-null or ad-BRCA1 before treatment with TNF α for 24 hours were quantified with a Human VEGF ELISA Kit (Thermo Scientific, Burlington, Ontario, Canada).

In Vitro Angiogenesis

The In Vitro Angiogenesis Assay Kit (Chemicon) was used to examine the angiogenic properties of ad-null- and ad-BRCA1-transfected HUVECs that had been incubated at 37°C in the absence or presence of TNF α . HUVECs were seeded onto ECMatrix Gel-coated 96-well plates (Millipore, Billerica, Mass) at a density of 9×10^3 /well. The extent of angiogenesis was determined with the aid of a phase-contrast microscope (Nikon, Tokyo, Japan) 5 hours post-plating. Each experiment was performed thrice in triplicates.

Migration Assay

Ad-null- and ad-BRCA1-transfected HUVECs were suspended in serum-free MCDB-131 medium and plated at a density of 0.25×10^6 /well, in the absence or presence of TNF α , in the upper chambers of a CytoSelect 24-well Cell Migration Fluorometric Assay system (Cell Biolabs, Inc). Ten percent fetal bovine serum complete MCDB-131 medium was added to the lower chambers and acted as the chemoattractant. After incubation for 12 hours at 37°C, the fluorescence emitted by the cells that had migrated to the lower chambers was quantified with the Thermo Scientific Fluoroskan Ascent FL Luminometer (ex/em: 480 nm/520 nm). Each experiment was performed thrice in triplicates.

Immunohistochemistry

Cryosections (5 μ m) of OCT-embedded murine aortic root and human carotid artery samples were subjected to immunohistochemical staining using standard methods and visualized with a DAB substrate (Vector Laboratories, Burlington, Ontario, Canada), and the degree of staining was quantified with the NIH ImageJ analysis software.

Endothelial Function and Hind-Limb Ischemia

Endothelial function in aortic segments from *EC-BRCA1*^{-/-} mice was studied via wire myography.

Unilateral hind-limb ischemia was induced in 8-week-old male BALB/c mice. The left femoral artery was ligated and excised distal to the origin of the deep femoral artery and proximal to the popliteal artery. Either ad-BRCA1 or ad-null (20 μ L of 10^{10} PFU/mL, intramuscularly) was administered on days 0 (immediately after surgery) and 14 at 5 different points in

the ischemic adductor muscle. Perfusion in the ischemic limb and the contralateral control limb was monitored by laser Doppler flow imaging before and immediately after surgery, and on postoperative days 4, 8, 16, and 28. On day 28, mice were euthanized, and the gastrocnemius muscles from the ischemic limbs isolated, embedded, and frozen in OCT cryosections (5 μ m) were stained with rhodamine-conjugated isolectin-B4 to aid in the identification of capillaries and with FITC-conjugated α -smooth muscle actin to discriminate arterioles. Capillary density, as defined by the number of isolectin-B4-positive areas over 5 to 7 microscopic fields, was determined using the NIH ImageJ software by an investigator blinded to the treatment groups.

Atherosclerotic Lesion and In Situ Reactive Oxygen Species Production

Eight-week-old male *ApoE*^{-/-} mice were fed the Western diet for 4 weeks and concurrently administered ad-GFP, ad-null, or ad-BRCA1 (20 μ of 10^{10} PFU/m) via the tail vein on days 0 and 14 of the study period. At the end of week 4, mice were euthanized and their aortic roots were isolated and embedded in OCT for cryosectioning (5 μ m). Sections were stained with BRCA1, Oil Red O, F4/80, or dihydroethidium (Molecular Probes, Burlington, Ontario, Canada) to determine the extent of BRCA1 expression, areas of atherosclerotic lesions, macrophage infiltration, and ROS production, respectively. Atherosclerotic lesions were identified by manually tracing the entire Oil Red O-positive areas and quantified with the NIH ImageJ analysis software. Macrophage infiltration was determined by coupling the F4/80 antibody to a DAB substrate. To measure ROS production, dihydroethidium (0.30 μ mol/L) was applied topically to each section. After 30 minutes of incubation at 37°C, sections were washed in PBS before they were overlaid with a coverslip. Fluorescent images of the sections were captured with a Nikon Eclipse E800 fluorescent microscope (Nikon, Tokyo, Japan) and quantified with the NIH ImageJ analysis software. A second group of *ApoE*^{-/-} mice were maintained on the Western diet for 16 weeks and concomitantly treated on day 0 and every second week thereafter with ad-null or ad-BRCA1 (20 μ L of 10^{10} PFU/mL). At the end of week 16, en face preparations of the whole aortas were stained with Oil Red O, and the stained areas were quantified with the NIH Image J analysis software. A third group of *ApoE*^{-/-} mice were fed the Western diet for 2 weeks, after which plasma samples were collected for evaluation of lipid levels through the in-house hospital laboratory services.

Statistical Analysis

Unless otherwise stated, data are presented as mean $\pm$ standard deviation for the number of samples. The Student *t* test was applied when the means of 2 groups were being compared. Differences between multiple means were evaluated by analysis of variance, and when overall differences were detected, individual means were compared post hoc with the Bonferroni's test.

FIGURE E1. BRCA1 overexpression in HUVECs and adenoviral transfection efficiency. A and B, Total RNA and protein were extracted from untransfected HUVECs and HUVECs that had been transfected 24 hours previously with ad-null or ad-BRCA1 (both 20 MOI). A, Representative image of an agarose gel showing the PCR products for *BRCA1*-specific and GAPDH primers with equal amounts of cDNA demonstrating basal expression of *BRCA1* in untreated and ad-null–transfected HUVECs, as well as *BRCA1* overexpression in ad-BRCA1–transfected HUVECs. B, Representative immunoblot supporting the transcript data in A at the protein level. GAPDH was used as a loading control. n = 3 to 4 in triplicate. C, Twenty-four hours post-transfection with 20 MOI of ad-GFP, approximately 80% of the HUVECs were positive for GFP staining. n = 3 to 4 in triplicate. *BRCA1*, BReast CAncer susceptibility gene 1; *ad*, adenovirus; *ad-BRCA1*, BRCA1 adenovirus; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *HUVEC*, human umbilical vein endothelial cell; *PCR*, polymerase chain reaction; *MOI*, multiplicity if infection; *cDNA*, complementary DNA; *GFP*, green flourescent protein.

FIGURE E2. Efficiency of adenoviral therapy and BRCA1 overexpression in *ApoE*^{-/-} mice. *ApoE*^{-/-} mice were treated with ad-GFP, ad-null, or ad-BRCA1 every 2 weeks while being fed the Western diet. A, Representative micrograph showing GFP fluorescence in the aortic root of an *ApoE*^{-/-} mouse treated with ad-GFP and fed the Western diet for 4 weeks. B, Representative micrograph showing BRCA1 positivity (*brown*) in the aortic root of an *ApoE*^{-/-} mouse treated with ad-BRCA1 and fed the Western diet for 4 weeks. C, Representative immunoblot with whole aortic lysates confirming endogenous expression of BRCA1 in ad-null-treated mice and BRCA1 overexpression in ad-BRCA1-injected mice. *n* = 3/group. *BRCA1*, BRCA1 susceptibility gene 1; *ad-GFP*, adenovirus green fluorescent protein; *ad-BRCA1*, BRCA1 adenovirus; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

TABLE E1. Lipid profiles of *ApoE*^{-/-} mice treated with ad-null or ad-BRCA1 and maintained on the Western diet for 2 weeks

Lipid (mmol/L)	ad-null (n = 8)	ad-BRCA1 (n = 7)
Total cholesterol	18.1 ± 3.31	16.6 ± 1.7
High-density lipoprotein cholesterol	0.64 ± 0.16	0.72 ± 0.21
LDL cholesterol	7.54 ± 1.6	7.94 ± 1.45
Very LDL cholesterol	7.61 ± 1.57	7.17 ± 0.81
Triglycerides	0.67 ± 0.16	0.77 ± 0.24

Data are presented as mean ± standard deviation. *BRCA1*, BRCA1 susceptibility gene 1; *LDL*, low-density lipoprotein; *ad*, adenovirus.