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Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system

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Abstract

Objective: Methylation of the promoter region of the estrogen receptor gene alpha (*ER α*) occurs as a function of age in human colon, and results in inactivation of gene transcription. In this study, we sought to determine whether such age-related methylation occurs in the cardiovascular system, and whether it is associated with atherosclerotic disease. **Methods:** We used Southern blot analysis to determine the methylation state of the *ER α* gene in human right atrium, aorta, internal mammary artery, saphenous vein, coronary atherectomy samples, as well as cultured aortic endothelial cells and smooth muscle cells. **Results:** An age related increase in *ER α* gene methylation occurs in the right atrium (range 6 to 19%, $R=0.36$, $P<0.05$). Significant levels of *ER α* methylation were detected in both veins and arteries. In addition, *ER α* gene methylation appears to be increased in coronary atherosclerotic plaques when compared to normal proximal aorta ($10\pm2\%$ versus $4\pm1\%$, $P<0.01$). In endothelial cells explanted from human aorta and grown in vitro, *ER α* gene methylation remains low. In contrast, cultured aortic smooth muscle cells contain a high level of *ER α* gene methylation (19–99%). **Conclusions:** Methylation associated inactivation of the *ER α* gene in vascular tissue may play a role in atherogenesis and aging of the vascular system. This potentially reversible defect may provide a new target for intervention in heart disease.

Keywords: DNA methylation; Receptors; Atherosclerosis; Aging; Hormones

1. Introduction

Heart disease is the leading cause of death in women. However, women are relatively protected against heart disease prior to menopause [1]. In observational studies estrogen replacement therapy is associated with a 40–50% reduction in the risk of heart disease in postmenopausal women, suggesting that estrogens are protective against atherosclerosis [2,3]. However, a recent randomized study in women with known coronary artery disease demonstrated no benefit to hormone replacement [4]. Interestingly, the lack of a protective effect of hormone replacement was observed in spite of changes in lipids that should have reduced the risk for heart disease. Hence, the study indicates that other mechanisms could have outweighed the beneficial effects of estrogens in reducing risk.

Estrogens exert their effects primarily via binding to the estrogen receptor (ER) which, once activated, regulates the

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transcription of multiple genes [5]. Although some of the potential protective effects of estrogens could be mediated by changes in the lipid profile, estrogens also have direct cardioprotective effects on the vessel wall [6,7]. In fact, functional ERs are present in the coronary arterial wall in both smooth muscle cells [8,9] and endothelial cells [10].

Evidence that the presence of ERs in the vessel wall may play an important role in protection against coronary atherosclerosis was provided by Losordo et al. [8]. Using monoclonal antibodies directed at the ER, these investigators found that coronary artery specimens obtained from premenopausal women with significant coronary atherosclerosis were less likely to stain for ERs than specimens from premenopausal women without coronary disease. This study thus established a possible link between lack of expression of the *ER* gene and atherosclerosis. Recently, an *ER*-like gene, human estrogen receptor β was identified [11]. However, its role in the cardiovascular system remains to be determined.

It is well established that a major mechanism for down regulation of gene expression is methylation of a cytosine and guanine rich area in the promoter region of the gene, called a CpG island. This promoter associated CpG island methylation has consistently been associated with permanent inactivation of gene transcription [12] in multiple systems. For example, this process is physiologically involved in inactivation of the X-chromosome [13] in which promoter methylation is essential to maintaining the silenced state, and where demethylation results in renewed gene expression. Promoter methylation is involved in genomic imprinting [14] in which the silenced state of the affected allele is determined by methylation of the promoter region for numerous imprinted genes, and demethylation results in bi-allelic gene expression. In addition, abnormal methylation of the promoter area of many genes appears to be an important feature of human neoplasia. Recently, CpG island methylation has been shown to be an alternative mechanism to mutation for inactivating tumor-suppressor genes [15], such as the Rb gene in retinoblastoma [16], the VHL gene in renal tumors [17] and the p16 gene in many neoplasms [18].

An example of the link between methylation and lack of expression of the estrogen receptor is methylation of the *ER* gene promoter in some ER negative breast cancers [19,20]. In these tumor cells, methylation of the promoter region of both *ER* alleles leads to inhibition of gene transcription. Methylation of the *ER* gene also occurs as a direct function of physiologic aging in normal human colonic mucosal cells [21]. In addition, methylation of the *ER* gene is present in virtually all colonic tumors. Thus, age-associated changes in *ER* gene methylation may be an early event which links aging with colon cancer.

Since coronary heart disease is also a disease of aging, and estrogens may be protective against coronary heart disease, we were interested in whether methylation of the *ER* α gene promoter occurs in the cardiovascular system. We also investigated whether *ER* α gene methylation is associated with atherosclerosis.

2. Methods

2.1. Tissue samples

Specimens were collected from men and women undergoing coronary artery bypass grafting (CABG), or directional coronary atherectomy (DCA) at Johns Hopkins Hospital. Specimens collected from CABG patients included the right atrial appendage (which was removed to place the patient on cardiopulmonary bypass), aortic punches (which were removed to connect the saphenous vein conduits to the aorta), as well as saphenous vein (SV) and internal mammary artery (IMA). Coronary atherosclerotic plaques were collected from patients undergoing DCA. Specimens were frozen and stored at −80°C until further processing. All patients gave informed consent for the use of their tissue samples. This study was approved by the Joint Committee for Clinical Investigation of the Johns Hopkins Hospital and Johns Hopkins University in accordance with the policies of the Department of Health and Human Services.

2.2. Detection of ER α gene methylation

Methylation of the CpG island of the *ER* α gene was detected using Southern blot analysis. The techniques used to process tissues, extract DNA and perform Southern blot analysis have been reported [21]. Briefly, specimens were homogenized in lysis buffer and incubated at 37°C overnight. DNA was isolated by phenol–chloroform extraction followed by ethanol precipitation, and resuspended in a trypsin–EDTA solution. For Southern blot analysis, 5 μg of DNA were incubated with the restriction enzymes EcoRI and NotI as specified by the manufacturer (NEB). EcoRI cuts a fragment that corresponds to the *ER* α promoter, and NotI is a methylation sensitive restriction enzyme. Following digestion the DNA was run on a 1% agarose gel and transferred to a nylon membrane (Zetaprobe, Bio-Rad). Filters were hybridized with a ^{32}P labeled *ER* α exon 1 probe and exposed in a Phosphorimager (Molecular Dynamics). Using IMAGEQUANT software (Molecular Dynamics), the percent methylation was calculated as the ratio of the density of the methylated uncut band (3.1 kb band in Fig. 1) over the total density of the three DNA fragments (3.1, 1.9 and 1.2 kb). To rule out incomplete digestion with the restriction enzymes, all blots were also probed with fragments of the thrombospondin and/or abl genes which are not methylated in normal tissues and should therefore be digested to completion.

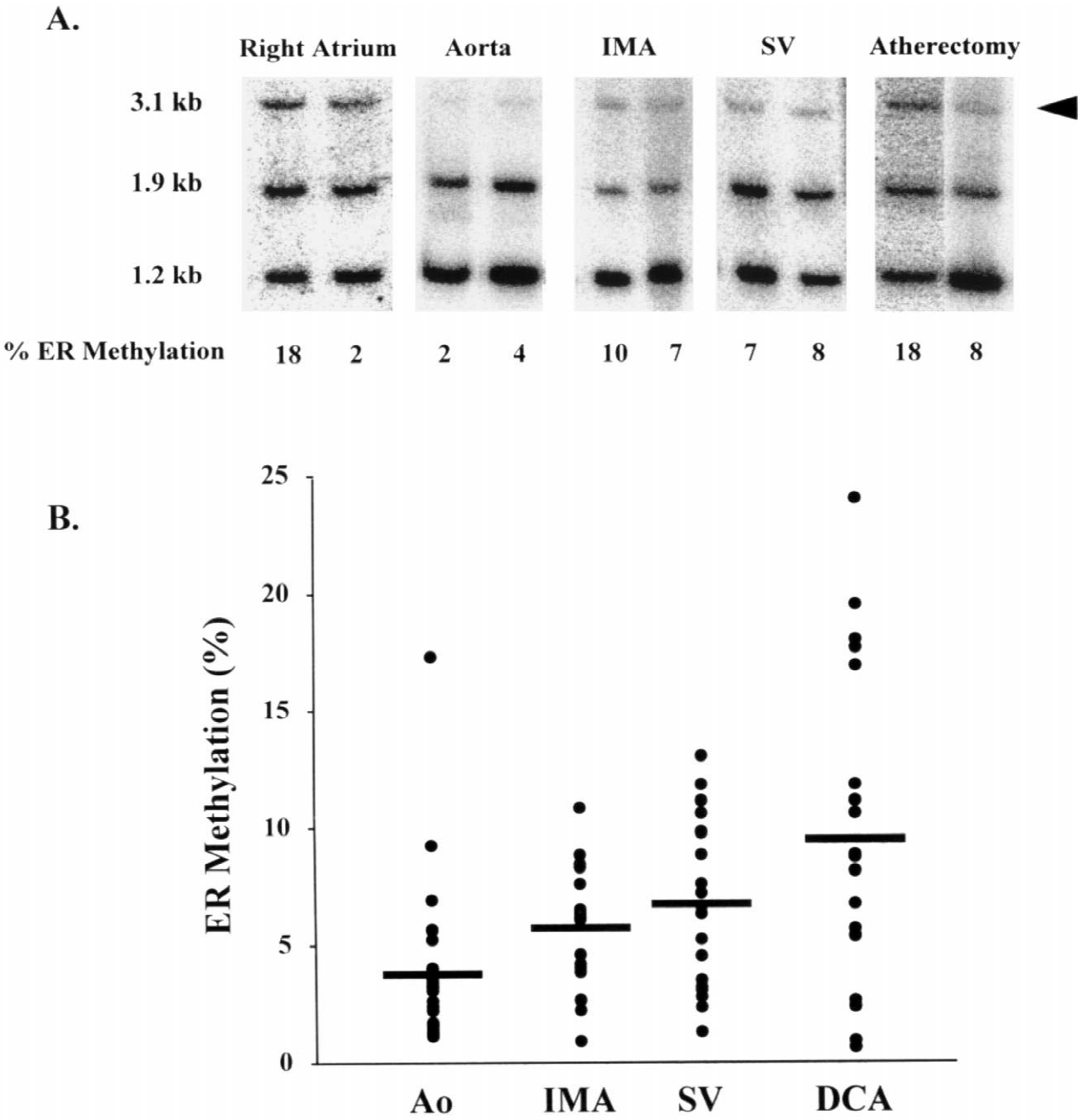

Fig. 1. Methylation of the *ER α* gene in cardiovascular tissue. (A) Representative Southern blot; DNA from the indicated samples was digested with EcoRI and NotI, run on an agarose gel and probed with exon 1 of the *ER α* gene. The 3.1 kb band (arrow) corresponds to the EcoRI flank cut, and indicates failure to cut with NotI due to methylation. The 1.9 kb and 1.2 kb are the normal (unmethylated) bands. The percent methylation was calculated as the ratio of the density of the methylated uncut band (3.1 kb band) over the total density of the three DNA fragments (3.1, 1.9 and 1.2 kb). (B) Comparison of *ER α* gene methylation in vascular samples. Shown are individual methylation values for all the samples analyzed from each tissue type. Each dot represents an individual sample, while the horizontal line represents the mean for each tissue type. *ER α* gene methylation in coronary atherectomy tissue was significantly increased when compared to macroscopically normal proximal aorta ($P<0.01$) or IMA ($P<0.04$): SV=saphenous vein, IMA=internal mammary artery, DCA=directional coronary atherectomy, Ao=aorta.

2.3. Cell culture

Human aortic endothelial cells and smooth muscle cells were purchased from Clonetics, and expanded in media with growth factors and antibiotics as recommended by the manufacturer. Expanded cultures were detached from their flasks by limited trypsin–EDTA treatment, aliquoted, pelleted and frozen until further analysis.

2.4. Statistics

Means, standard errors, and linear correlations were calculated with EXCEL software (Microsoft). Student's *t*-test was used to measure differences in continuous variables using EXCEL. Multiple linear regression was used to calculate the age-adjusted difference between right atrial *ER* methylation in men and women using SAS software.

3. Results

3.1. Methylation of the ER α gene in cardiovascular tissues

We measured *ER α* gene methylation in human aorta, IMA, SV and right atrium. All tissues were collected from individuals undergoing coronary artery bypass grafting. The degree of methylation of the promoter region of the *ER α* gene was measured by Southern blot analysis (examples in Fig. 1a, summary in Fig. 1b). *ER α* gene methylation was consistently detectable in all tissues examined. In macroscopically normal aortic specimens from twenty men and seven women, *ER α* methylation ranged from 1 to 18% (mean 4±1%). *ER α* methylation was similarly detectable in IMA samples from ten men and nine women (range 1 to 11%, mean 6±1%) and in SV samples from thirteen men and eight women (range 2 to 13%, mean 7±1%). SV samples had significantly higher methylation than aortic samples ($P<0.01$) but the difference between aorta and IMA or SV and IMA did not reach statistical significance. *ER α* methylation was independent of age and gender in the aorta, IMA and SV (overall, mean *ER α* methylation was 5.4% in 43 samples from men and 5.3% in 24 samples from women). *ER α* methylation was measured in the right atria of 17 men and 14 women (range 34 to 88 years old). This included two premenopausal women and one postmenopausal women who was receiving hormone replacement therapy. Methylation of the *ER α* gene varied between 6 and 19% (mean 11±1%) (Fig. 1). A significant linear correlation between *ER α* gene methylation and age was present in these samples ($r=0.36$, $P<0.05$). In the right atrium, there was no difference in *ER α* gene methylation between men and women. These data suggested that promoter methylation does occur in cardiovascular tissues and affect blood vessels in a non-uniform mosaic way.

3.2. Increased methylation of the ER gene in directional coronary atherectomy samples

Given the data that methylation of the *ER α* gene in some tissues of the cardiovascular system is linked to aging, and that age is a significant factor in the process of atherogenesis, we hypothesized that the level of methylation of the *ER α* gene in coronary atherosclerotic tissue might be elevated. Coronary atherosclerotic plaques were collected from twelve men and seven women undergoing directional coronary atherectomy. In these samples, *ER* gene methylation varied from 1 to 24% (mean 10±2%) (Fig. 1) and was not related to age or gender. Since it is not possible to obtain normal coronary artery specimens, the macroscopically normal aortic 'punch' and internal mammary arteries are the best surrogates available for a 'normal' artery. *ER α* gene methylation in coronary atheromas was significantly increased when compared to macroscopically normal proximal aorta ($P<0.01$) or IMA ($P<0.04$). Thus, a significant proportion of atherosclerotic plaques display increases in *ER α* methylation which may contribute to the previously observed lack of *ER α* expression in these lesions [8].

3.3. Methylation of the ER α gene in early passages of cultured endothelial and smooth muscle cells

Vascular and atherectomy samples contain several types of nucleated cells, including endothelial cells and smooth muscle cells, and also a few fibroblasts, monocytes, lymphocytes and neutrophils. To identify the cell type which might be contributing to *ER α* gene hypermethylation in atherectomy samples, we studied pure populations of cells grown in vitro.

Human aortic endothelial cells (HAEC) and human aortic smooth muscle cells (HASMC) obtained from cadavers (Clonetics) were examined for *ER α* gene methylation, with Southern blot analysis, at the earliest passage available. *ER* gene methylation was consistently low in the six cultured HAEC lines examined (1–4%, see Fig. 2). In contrast to the data obtained with HAEC, the cultured HASMC had consistently high levels of *ER α* gene methylation at the earliest passage examined in the four cell lines analyzed (Fig. 2). Methylation ranged from 19% in one sample obtained from an infant, to 99% in a sample obtained from an adult patient. Since normal aorta, which consists primarily of smooth muscle cells, has very little *ER* methylation (see above), these results suggest that those few aortic smooth muscle cells carrying methylated *ER* alleles might be selected for growth in vitro under cell culture conditions, and, possibly, in vivo in atherosclerotic plaques.

4. Discussion

Methylation of the promoter region of the *ER* gene is associated with inhibition of gene transcription in several cell systems. We have now shown that methylation of the *ER α* gene is present in a non-uniform, mosaic, distribution in the cardiovascular system. *ER α* gene methylation can be found at variable levels in all vascular tissues studied, including the right atrium, saphenous veins, IMA, and the proximal aorta. An age related increase in *ER α*

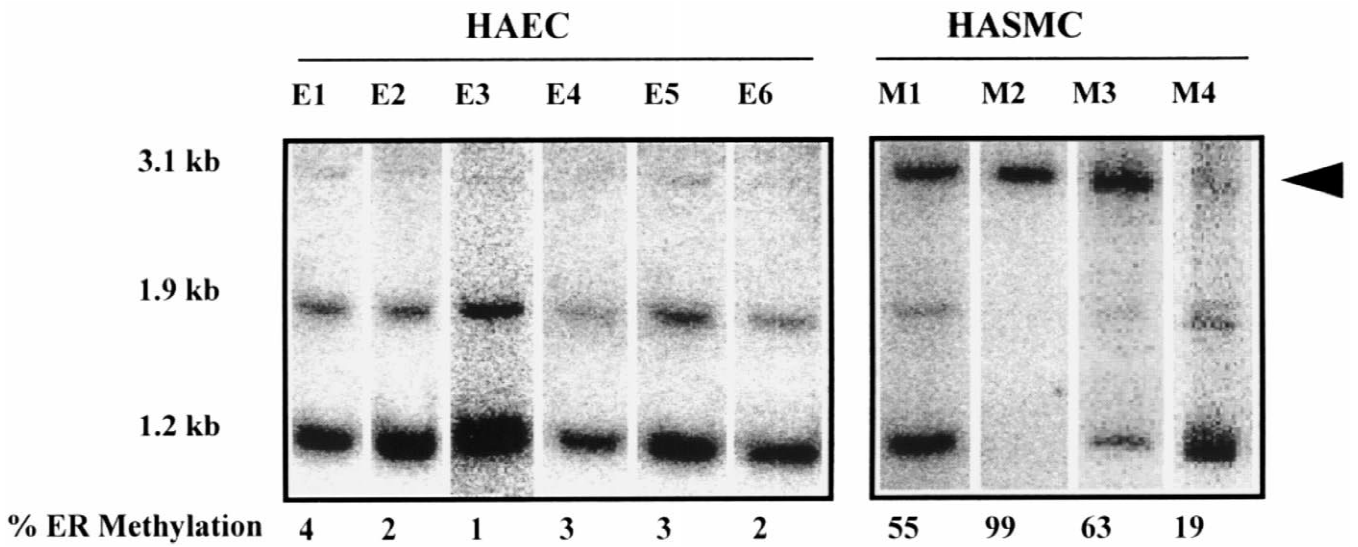

Fig. 2. Methylation of the *ER* gene in the earliest passages available of cultured vascular cells. Representative Southern blot of several HAEC and HASMC cell lines. DNA from the respective cells was studied as in Fig. 1. The name of each cell line is indicated on top of each lane. The arrow points to the methylation band at 3.1 kb. The value for percentage *ER* methylation is indicated on the bottom of the Southern blots. HAEC=human aortic endothelial cells, HASMC=human aortic smooth muscle cells.

gene methylation occurs in the right atrium, and a higher degree of methylation is present in coronary atheromas compared to macroscopically normal vascular tissues (aorta and IMA).

Proliferating HASMC in culture have a high level of *ER* α gene methylation in sharp contrast with the *ER* α in cultured HAEC which is nearly unmethylated. Current methodology does not allow separate analysis of gene methylation between HASMC and HAEC in vivo. Nevertheless, our results are consistent with the possibility that proliferation of abnormally methylated smooth muscle cells may contribute to the increased methylation found in diseased cardiovascular tissues. Remarkably, cultured primary smooth muscle cells display a phenotype similar to that observed in the same cells retrieved from expanding plaques in atherosclerotic coronary vessels [22]. Since the vascular tissues we evaluated contain several different cell types, a change in methylation from 4 to 10% may represent a substantial change in gene inactivation if a few cells have high levels of methylation. The results of our cell culture experiments suggest that the smooth muscle cells might be the cells preferentially methylated and selected for growth in atherosclerotic plaques. A change from 4 to 10% *ER* methylation may therefore mean a 2.5-fold increase in the number of proliferating SMCs which may contribute to atherogenesis.

Estrogens have been shown to directly inhibit SMC proliferation [6,23–25]. Thus, it is possible that the high degree of methylation of the *ER* α gene in cultured HASMC results from the selective loss of growth control such methylation would confer upon the cells. Alternatively, the presence of an active *ER* α gene, might represent a negative selection factor, thereby promoting the growth of surviving cells that had previously acquired methylation of their *ER* α gene. That this methylation arises in vivo is evidenced by the relatively high level of methylation in some atheromatous tissues, where abnormal SMC proliferation can be seen. Overall, our data is consistent with a model whereby aging and, perhaps vascular injury is associated with de novo methylation of the *ER* α gene promoter in some SMCs. These cells lose some of the normal growth control imparted by circulating hormones. Under certain conditions, these cells proliferate abnormally and may contribute to the pathogenesis of the atheromatous plaque. Alternatively, it is also possible that the *ER* α gene methylation we have observed is secondary to the changes that lead to atherosclerosis and hyperproliferation in vivo and in vitro. Experiments aimed at modulating the levels of *ER* α gene methylation and observing its effects on atherosclerosis and SMC proliferation should distinguish these possibilities.

Previous studies of CpG island promoter methylation, in multiple settings, including inactivation of the X-chromosome, genomic imprinting, and many tumor-suppressor genes, have consistently been associated with permanent inactivation of gene transcription [12]. In fact, a close association between methylation and repressed *ER* gene expression has been demonstrated in multiple diseased tissues including breast cancer, colorectal cancer and hematopoietic neoplasm. Consist with this known association, our preliminary studies, using Western blot analysis of *ER* α expression in cultured HASMC and HAECs, reveal the presence of *ER* α expression in the unmethylated HAECs and lack of expression in the strongly

methylated HASMCs (data not shown). Thus it appears reasonable to assume that, in SMCs, the degree of methylation observed would be correlated with a marked down-regulation of expression of the gene.

Recent epidemiological studies demonstrate that estrogen replacement therapy may be one of the most potent anti-atherosclerotic therapies available for postmenopausal women [2,3]. Some of the effects of estrogens on the vessel wall include improvement in endothelial dysfunction [7] (as demonstrated by reversal of paradoxical acetylcholine induced vasoconstriction), prevention of endothelial cell apoptosis [26], and inhibition of vascular SMC proliferation [6,23–25]. However, a recent randomized study of hormone replacement therapy in postmenopausal women with coronary artery disease (HERS study) revealed no reduction in cardiac deaths or MI in women receiving hormone therapy compared with placebo [4]. The higher degree of methylation of *ER α* in a subpopulation of cells of atherosclerotic tissue compared to normal aorta supports our hypothesis that the lack of expression of the *ER α* gene in SMCs, resulting in an inability to respond to estrogen's protective effects, may be an integral step in the development of the atherosclerotic lesion. This methylation in diseased vessels may also inhibit the potential protective effects of estrogen in women with advanced atherosclerotic disease, and therefore, account, at least in part, for the lack of benefit observed in the HERS study. Ongoing randomized trials of hormone replacement therapy using angiographic outcomes may provide further insight as to the potential link between *ER α* methylation and vascular effects of estrogen.

The results of this investigation may have important clinical implications. The presence of a high level of *ER α* gene methylation in some women, particularly at the level of some vascular sites may partially negate the benefit of estrogen replacement in these women. In addition, *ER α* gene methylation is potentially reversible pharmacologically, using inhibitors of the DNA-methyltransferase enzyme [20]. Further studies are needed to determine the predictors of *ER α* gene methylation in humans, and the impact of its prevention or reversal using demethylating agents. Such approaches could be relevant for the control of SMC proliferation in pathological processes such as plaque formation and restenosis post-angioplasty.

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