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ET POSTDOCTORALES**



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**Estrogen Receptor Beta and Atherogenesis:  
Role of Receptor Related NM23-H2 and HSP27**

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**Estrogen Receptor Beta and Atherogenesis: Role of Receptor Related Proteins**

**NM23-H2 and HSP27**

by

**KATEY RAYNER**

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial  
fulfillment of the requirement for the degree of

**DOCTOR OF PHILOSOPHY**

Department of Biochemistry, Microbiology and Immunology

University of Ottawa

Ottawa, Ontario, Canada

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## **Please note**

- 1-** Figure 2.1C, p. 64 should be p. 67
- 2-** p. 67 is repeated twice
- 3-** Figure 4.8, p. 140, should be p. 141
- 4-** p. 140 is repeated twice
- 5-** missing pages 195 & 196

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Of course nothing that is presented in this thesis would have been possible without the wonderful support, training and mentorship I received from so many individuals throughout my graduate training. When I arrived at the Heart Institute in 2003, I was immediately welcomed by members of the O'Brien lab who taught me so many skills and techniques that eventually proved to be essential to my research. Over my five years spent in the lab, members both past and present have contributed in some fashion to my research, and I am truly indebted to each of them. I would especially like to thank the current members in the lab Dr. Yong-Xiang Chen, Dr. Kevin Sun and Melissa McNulty, who contributed significantly to this work. I am also grateful to many individuals at the Heart Institute, particularly members of Stewart Whitman's lab, for the support and assistance throughout my graduate career. On a personal level, I have been tremendously lucky to be supported by my husband Alistair through all the ups and downs that accompany graduate school. My parents and sisters have also been an amazing source of inspiration and encouragement and I thank them for making me both the person and the scientist I am today. Last but certainly not least I owe so much of my success to my supervisor and mentor Ed O'Brien. His support for my ideas, his encouragement of my independence, and his sense of humour throughout my entire graduate career have made me the scientist I am today, and for that I am very grateful.

## ABSTRACT

There exists an obvious gender disparity in the incidence of cardiovascular disease, with more women over the age of 60 years dying each year compared to their male counterparts. However, pre-menopausal women appear to be spared from developing this disease. Naturally occurring female hormones such as estrogen are thought to provide the cardiovascular system with some form of protection from atherosclerosis. However, the mechanisms behind this protection are poorly understood, and large clinical trials failed to demonstrate any significant cardiovascular benefit for women receiving hormone replacement therapy. Estrogen acts through two primary receptors, ER $\alpha$  and ER $\beta$ , and evidence is emerging to suggest that ER $\beta$  is a key mediator of estrogen action in the vessel wall. The **objective** of this doctoral thesis was to understand more about ER $\beta$  in atherosclerosis by elucidating novel molecular mechanisms of ER $\beta$  action. Two novel proteins were discovered to interact with ER $\beta$  in the vessel wall: (i) non-metastatic protein-23 (NM23-H2) and (ii) heat shock protein 27 (HSP27). The first portion of this study demonstrated that NM23-H2 and ER $\beta$  interact in vascular cells *in vitro* and NM23-H2 can act as a modest co-activator of estrogen-mediated transcriptional signaling via ER $\beta$ . The expression of NM23-H2 is diminished with progression of atherosclerosis in human coronary arteries, indicating that with advancing disease, the regulation of important estrogen and ER $\beta$ -mediated events may be impaired. The second portion of this study showed that over-expression of HSP27 in a mouse model of atherosclerosis can protect from the development of atherosclerotic lesions, but is dependent on the presence of estrogen. HSP27 release from macrophages

is induced by estrogen, where it may serve to protect against atherosclerosis by binding the scavenger receptor-A and preventing the uptake of atherogenic lipoproteins and the ensuing inflammation. The release of this atheroprotective HSP27 is mediated preferentially by ER $\beta$  both *in vitro* and *in vivo*, uncovering a novel mechanism of ER $\beta$ -mediated protection in the vessel wall. In summary, this doctoral work reveals two novel ER $\beta$ -associated proteins that can modulate the response to estrogen both *in vitro* and *in vivo*, and may be the target of future therapeutics designed to treat and prevent cardiovascular disease in both men and women.

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### LIST OF ABBREVIATIONS

|             |                                             |               |                                                  |
|-------------|---------------------------------------------|---------------|--------------------------------------------------|
| ANOVA       | analysis of variance                        | HFD           | high-fat diet                                    |
| acLDL       | acetylated LDL                              | HRT           | hormone replacement therapy                      |
| apoE        | apolipoprotein E                            | HSP27         | heat shock protein 27                            |
| AR          | androgen receptor                           | HSP60         | heat shock protein 60                            |
| BSA         | bovine serum albumin                        | HSP70         | heat shock protein 70                            |
| CAD         | coronary artery disease                     | HSP90         | heat shock protein 90                            |
| CEE         | conjugated equine estrogen                  | ICI           | ICI 182,170                                      |
| CVD         | cardiovascular disease                      | IgG           | immunoglobulin G                                 |
| DBD         | DNA binding domain                          | IL-10         | interleukin-10                                   |
| DTSSP       | 3,3'-Dithiobis(sulfosuccinimidylpropionate) | IL-1 $\beta$  | interleukin-1 beta                               |
| DMEM        | Dulbecco's modified eagle medium            | IL-6          | interleukin 6                                    |
| DPN         | diarylproprionitrile                        | IP            | immunoprecipitation                              |
| E2          | estrogen                                    | kDa           | kiloDalton                                       |
| E2          | 17-beta estradiol                           | LAD           | left anterior descending                         |
| EC          | endothelial cell                            | LAMP          | lysosome associated membrane protein             |
| ECFP        | enhanced cyan fluorescent protein           | LCX           | left circumflex                                  |
| EDTA        | ethylenediaminetetraacetic acid             | LDH           | lactose dehydrogenase                            |
| EGFP        | enhanced green fluorescent protein          | LDL           | low density lipoprotein                          |
| EGTA        | ethylene glycol tetraacetic acid            | LDLR          | LDL receptor                                     |
| ELISA       | enzyme-linked immunosorbent assay           | LPS           | lipopolysaccharide                               |
| eNOS        | endothelial nitric oxide                    | MAPK          | mitogen activated protein kinase                 |
| ERE         | estrogen response element                   | MBP           | maltose-binding protein                          |
| ER $\alpha$ | estrogen receptor alpha                     | MCP-1         | monocyte chemoattractant protein-1               |
| ER $\beta$  | estrogen receptor beta                      | MI            | myocardial infarction                            |
| EU          | endotoxin unit                              | MMP           | matrix metalloproteinase                         |
| FBS         | fetal bovine serum                          | MW            | molecular weight                                 |
| FHS         | Framingham Heart Study                      | NBF           | neutral buffered formalin                        |
| HBD         | hormone binding domain                      | NDPK          | nucleoside diphosphate kinase                    |
| HDL         | high density lipoprotein                    | NF $\kappa$ B | nuclear factor kappa-light-chain-enhancer of act |
| HERS        | Heart Estrogen/Progestin Replacement Study  | NHS           | Nurses' Health Study                             |

|              |                                            |
|--------------|--------------------------------------------|
| NM23         | non-metastatic protein 23                  |
| NO           | nitric oxide                               |
| NR           | nuclear receptor                           |
| OVX          | ovariectomy                                |
| oxLDL        | oxidized low density lipoproteins          |
| PAGE         | polyacrylamide gel electrophoresis         |
| PBS          | phosphate buffered saline                  |
| PFA          | paraformaldehyde                           |
| PMA          | phorbol 12-myristate 13-acetate            |
| PPT          | propylpyrazole-(1,3,5-triyl)trisphenol     |
| PMSF         | phenylmethanesulphonylfluoride             |
| PVDF         | Polyvinylidene Fluoride                    |
| RCA          | right coronary artery                      |
| ROS          | reactive oxygen species                    |
| RT           | room temperature                           |
| SDS          | sodium dodecyl sulfate                     |
| SERM         | specific estrogen receptor modulator       |
| SMC          | smooth muscle cells                        |
| SR-A         | scavenger receptor-A                       |
| SRC          | steroid receptor coactivator-1             |
| TAF          | transcriptional activation function domain |
| TNF $\alpha$ | tumor necrosis factor alpha                |
| VCAM         | vascular adhesion molecules                |
| VEGF         | vascular endothelial growth factor         |
| WHI          | Women's Health Initiative                  |
| Y2H          | yeast two hybrid                           |

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## Chapter 2

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Katey Rayner, Yong-Xiang Chen, Benjamin Hibbert, Dawn White, Harvey Miller, Edith H. Postel and Edward R. O'Brien. *NM23-H2, an Estrogen Receptor  $\beta$  Associated Protein, Shows Diminished Expression with Progression of Atherosclerosis*

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#### **Manuscript #2**

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### **Manuscript #3**

Katey Rayner\*, Yong-Xiang Chen\*, Melissa McNulty, Trevor Simard, Xioaling Zhao;  
Dominic J. Wells, Jacqueline de Belleruche, Edward R. O'Brien; *Extracellular Release  
of the Atheroprotective Heat Shock Protein 27 is Mediated by Estrogen and  
Competitively Inhibits acLDL Binding to Scavenger Receptor-A*

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## **1.0 INTRODUCTION**

Vascular diseases are the most prominent cause of morbidity and mortality in the modern world, leading to heart attacks, strokes, and heart failure. Atherosclerosis is defined as a hardening of the arteries, which leads to narrowing of the blood vessel lumen and the restriction of blood flow to surrounding tissues. Historically, the underlying cause of atherosclerosis centered on the presence of high levels of cholesterol in the circulation, but over the past decade this paradigm has shifted; it is now widely recognized that this disease is caused by a state of unwanted chronic inflammation within the blood vessel wall that persists throughout adulthood. The current understanding of the pathophysiology of atherosclerosis suggests that this disease is initiated at a relatively young age, and yet goes undetected until it manifests itself later in life, often in the form of a fatal myocardial infarction (MI). A better appreciation for the detailed molecular mechanisms of atherosclerosis will allow for improved prevention and treatment strategies that will affect millions of individuals worldwide.

### **1.1 The Cardiovascular System In Health and Disease**

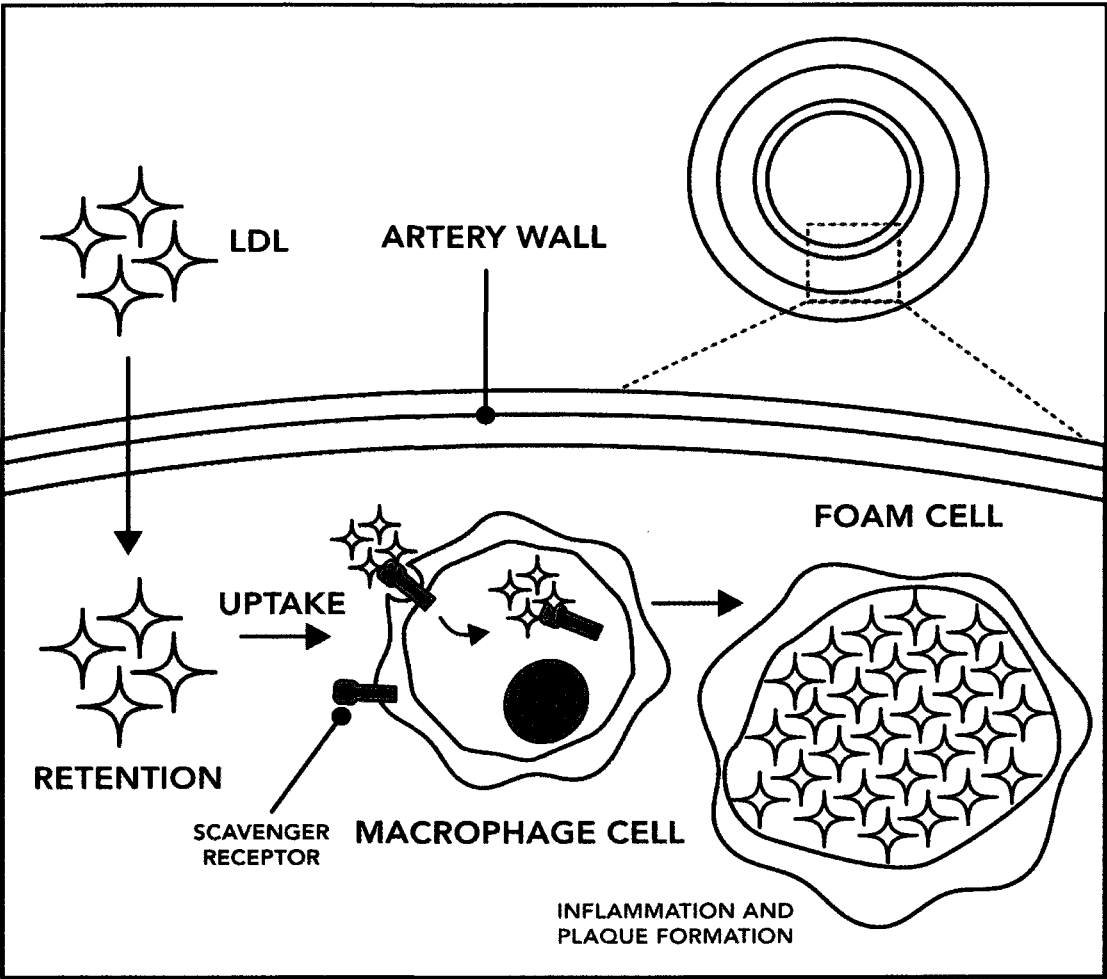
#### **1.1.1 Initiation of Atherosclerosis**

Blood vessels are composed of three major layers: (i) an endothelium, made up of a monolayer of endothelial cells (ECs), separating the circulating blood from the underlying tissues; (ii) a medial layer, consisting of contractile smooth muscle cells (SMCs); and (iii) an adventitia, composed mainly of collagen and fibroblasts, providing support to the artery wall and anchoring it to the surrounding tissue (40). In a healthy vessel, the space between the

endothelium and the medial SMCs contains the internal elastic lamina (IEL), which in part is responsible for the elastic properties of the artery. At the very first stage of atherosclerosis initiation, low-density lipoprotein (LDL) particles deposit beneath the endothelium, usually as a result of high levels of circulating cholesterol (one of the major risk factors for atherosclerosis). Due to its interaction with negatively charged components of the extracellular matrix (e.g. proteoglycans) this LDL becomes trapped in the subendothelial space, and cannot egress back into the circulation (127). Due to a variety of local factors, including the presence of resident free radicals and absence of antioxidants, LDL that is deposited in the vessel wall can become oxidized (**Figure 1.1**) (121). Intimal oxidized LDL (oxLDL) signals to the circulating immune cells to be recruited to the subendothelial space, with monocytes being the first cells to respond to these signals (**Figure 1.2**) (43). This accumulation of macrophages and cholesterol beneath the endothelium is termed the atheromatous plaque- the hallmark feature of atherosclerosis. At the earliest stages of disease, the plaque accumulates into a fatty streak, and is the first pathological step in the progression of atherosclerosis and the first histologically discernable stage of disease (**Figure 1.3**) (104). Macrophages recruited to the plaque attempt to clear the unwanted cholesterol deposits by engulfing them using scavenger receptors present on their cell surface, and as macrophages become engorged with lipid, they take on a foamy appearance, and are hence termed foam cells. Scavenger receptors, including SR-A and CD36, normally recognize and clear harmful pathogens from healthy tissue; however, in the setting of atherosclerosis, they recognize oxLDL as a non-self pathogen and initiate an unwanted inflammatory response (14,66,96). Unlike the LDL receptor, SR-A and CD36 are not downregulated by increased cellular cholesterol content, and macrophages become loaded with lipid and appear trapped in the vessel wall, where they readily secrete pro-inflammatory

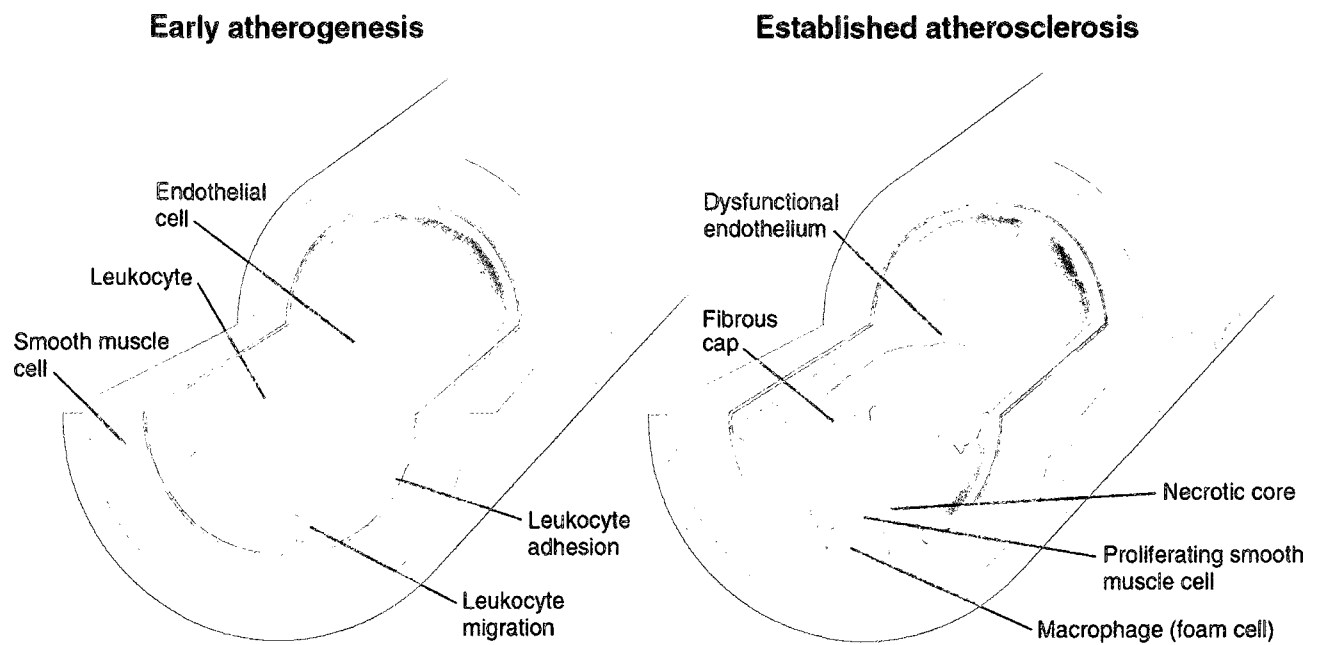
**Figure 1.1: Mechanisms of atherosclerosis initiation.** Low-density lipoprotein (LDL) can become oxidized and trapped within the vessel wall, leading to recruitment of macrophages beneath the endothelium and inflammatory foam cell formation. Cholesterol and foam cells are the main components of fatty streaks- the earliest discernable atherosclerotic plaques.

**Figure 1.1**



**Figure 1.2: Mechanisms of atherosclerosis propagation.** Once macrophages migrate into the vessel wall and take up oxLDL, they activate inflammatory pathways, secreting cytokines, chemokines, proteases and free radicals into the extracellular space. This recruits additional monocytes to the site of inflammation, propagating disease and furthering tissue damage, including damaging the endothelium. SMCs migrate into the intimal layer, and over time a fibrous cap forms around the necrotic core.

**Figure 1.2**

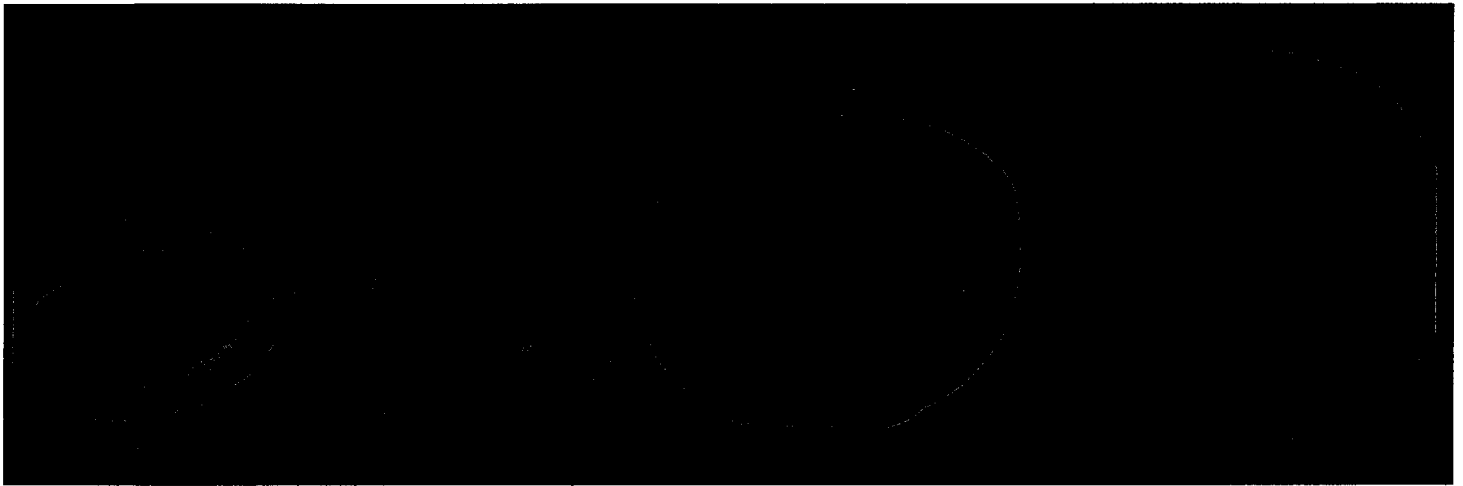




**Figure 1.3: Human coronary arteries with various stages of atherosclerosis.** Cross sections of human coronary artery specimens showing the gross morphology of progressing stages of atherosclerosis. From left to right: (i) normal artery, showing a normal lumen size; (ii) fatty streak, showing plaque development and compensatory outward remodeling and thickening of the vessel wall; (iii) atheroma, showing cholesterol deposits beneath the endothelium and large plaque growth with narrowed lumen area; (iv) complex plaque showing accumulated cholesterol and cellular debris within the plaque covered by a stable fibrous cap, and considerable narrowing of the lumen area.

**Figure 1.3**

(i) normal      (ii) fatty streak      (iii) atheroma      (iv) complex plaque



cytokines (i.e. IL-1 $\beta$ , TNF- $\alpha$ ), chemokines (i.e. MCP-1) and reactive oxygen species (reviewed in 83, 127). Consequently, blocking scavenger receptor-mediated uptake of oxidized lipids was proposed as a means to abrogate foam cell formation and atherosclerosis. Under normal conditions, ECs prevent the migration of inflammatory cells from the circulation to the intimal or medial layer of the vessel wall; however, local inflammation causes ECs to express adhesion molecules and chemoattractant molecules that recruit additional monocytes and lymphocytes to the site of oxLDL accumulation (103). Intimal foam cells also signal to SMCs to lose their normally quiescent, contractile phenotype and become activated, engulfing oxLDL and migrating into the intimal area, releasing proteases and extracellular matrix components en route (100). This subclinical atherosclerosis can persist undetected for many years, yet it is these fatty streaks that have the potential to develop into large complex plaques that are risk factors for future clinical events.

### **1.1.2 Progression and Complications of Atherosclerosis**

Over time, as the plaque continues to expand, the luminal area of the vessel becomes smaller and blood flow can become restricted. When this disease is located in the coronary circulation, the myocardium becomes ischemic and is accompanied by symptoms such as angina. Often the growing atherosclerotic plaque is contained within a protective fibrous cap, comprised of SMCs and extracellular matrix proteins, which protects the plaque from proteases present in the circulation. The fatty streak progresses into a complex atherosclerotic lesion by the deposition of collagen and calcium, primarily secreted by the medial SMCs that have migrated into the plaque over time. As the complex plaque continues to expand, macrophages and SMCs beneath the fibrous cap cannot handle the

burden of excess cholesterol present in the atheroma; they become necrotic and release their contents (i.e. esterified cholesterol, cytokines and proteases) into the surrounding space (19,20). This results in the development of a necrotic core within the plaque, histologically represented by a paucity of SMCs and large cholesterol clefts contained beneath the fibrous cap (110). The necrotic debris released by these cells then re-initiates the inflammatory cascade, recruiting additional cells to the site of inflammation and the release of proteases such as matrix metalloproteinase-9 leading to the destruction of the surrounding connective tissue and the weakening of the fibrous cap (92). Once the fibrous cap has become sufficiently degraded, it can rupture and expose the inner contents of the plaque to the circulation, where it comes in contact with thrombotic elements like platelets and fibrin, initiating the formation of a thrombus within or on the surface of the plaque (31,32). Thrombi can cause the complete occlusion of the artery, causing dramatic ischemia to the downstream tissues and ultimately lead to myocardial infarction or even death.

### **1.1.3 Models of atherosclerosis**

Many advances concerning our understanding of the mechanisms of atherosclerosis have come as a result of mouse models. Rarely do mice spontaneously develop atherosclerosis, in part due to their high levels of HDL and low levels of LDL (133). However, by perturbing normal cholesterol metabolism, by either genetically knocking out apolipoprotein E (a key component of LDL) or the LDL receptor (necessary for clearance of lipid) mice become hypercholesterolemic and can develop atherosclerosis. The apoE<sup>-/-</sup> mice cannot easily clear LDL from the circulation, leading to LDL depositing in the vessel wall and more susceptible to oxidation (133). LDLR<sup>-/-</sup> mice are also deficient in removing cholesterol from the

circulation, though less hyperlipidemic than apoE<sup>-/-</sup> mice and require high-fat diet to induce atherosclerosis. In both mouse models, within weeks of being placed on a high-fat (or ‘Western’) diet, inflammatory atherosclerosis ensues (i.e. macrophages infiltrate the vessel wall in response to cholesterol deposits, leading to large, complex inflammatory lesions, much like those observed in humans). These mice are invaluable for our understanding of how atherosclerosis develops *in vivo*, and by combining these models with knock-out and/or transgenics of other molecules, have provided us with a platform on which to examine genes involved in disease progression.

Overall, inflammation plays a pivotal role throughout all stages of atherosclerosis development, from fatty streak initiation to necrotic core formation and plaque rupture. The main cellular player is the macrophage, which fails to rectify the accumulation of inflammatory lipoproteins, ending up exacerbating the disease process. Targeting this cell type for therapeutic purposes is therefore desirable, both for prevention of inflammatory plaque development in the early disease process, and for decreasing foam cell formation at later stages of the disease.

## **1.2 Cardiovascular Disease and Women’s Health**

More women in Canada die as a result of heart disease than any other cause (119). However, a woman’s risk of developing atherosclerosis is relatively low until menopause, from which point the risk is greater than that of men (81,129). In the absence of endogenous estrogen (i.e. after menopause) there is an overall increase in the risk factors for coronary disease, including an increase in LDL, a decrease in HDL, and an increase in hypertension. Although

estrogen has effects on these traditional risk factors, it is believed that this accounts for less than a third of the overall protective benefits that are observed before menopause, implying estrogen must have additional beneficial effects that act directly on the vessel wall (discussed below) (81). Overall, it appears that estrogen is protective, and replacing estrogen in post-menopausal women would have favourable effects on cardiovascular health. However, as will be discussed, hormone replacement therapy (HRT) was not the panacea it was expected to be, and more information is still required about how estrogen acts in the vessel wall.

### **1.2.1 Clinical Evidence of Estrogen Protection**

The observation that pre-menopausal women have lower risks of coronary artery disease (CAD) than men prompted the investigation into the role of ovarian hormones in maintaining cardiovascular health. Some of the first evidence concerning the protective effects of estrogen on the vascular system came from the Nurse's Health Study (NHS), a groundbreaking observational study of over 120,000 nurses aged 30 to 55 years old in the United States (8). The NHS found that there was a 30% reduction in risk of developing coronary heart disease (as determined by incidence of MI) in women who used hormone replacement therapy after menopause compared to those that did not. A ten-year follow-up of >48,000 NHS participants with no history of cancer or cardiovascular disease demonstrated an even greater 54% reduction in risk of MI for women currently using estrogen therapy compared to those who were not (118). However, the Framingham Heart Study (FHS) suggested that hormone replacement therapy may increase the risk of developing MI or stroke (129). The disparate conclusions of these observational studies led to another seminal study in women's health: the Women's Health Initiative (WHI) trial. This large-scale, randomized, placebo-controlled trial directly examined the effect of HRT on cardiovascular events in over 16,000

women aged 50-79 years old. The conclusion from this trial was that the use of HRT (in the form of conjugated equine estrogen, CEE) was associated with an overall 24% increased risk in cardiovascular events for women using CEE compared to those on placebo (**Figure 1.4**) (73). The evidence was so strong that not only was the WHI trial terminated prematurely due to safety concerns, but it led to the widespread stoppage of HRT in women world-wide. Similarly, the HERS trial (Heart Estrogen/Progestin Replacement Study) demonstrated that in over 2,000 women with existing coronary artery disease, HRT did not provide any protection from death due to MI or even reduction in other cardiovascular events (58). Many of the women receiving HRT in the HERS trial experienced an increase in venous thromboembolic events, suggesting that not only does estrogen not provide cardiovascular benefits, but it may *increase* risk of life-threatening events. The contradictory findings between the observational studies and the clinical trials for both primary and secondary prevention of CAD highlight the overall lack of understanding of the underlying effects of estrogen on the vessel wall. Recently, the American Heart Association conducted an evaluation of heart disease with respect to women's health and concluded that, as researchers, it was imperative to: (i) determine the mechanism of estrogen on cardiovascular events in the first year of HRT (when risk of adverse events is highest), and (ii) understand more about the beneficial effects of endogenous estrogens (126). This underscores the importance of further study of estrogen in atherosclerosis, and is the focus of this doctoral thesis.

**Figure 1.4: Summary of Negative Findings of the WHI Trial.** Women in the Women's Health Initiative (WHI) Trial who received estrogen + progestin had higher incidence of coronary heart disease (CHD) than women receiving placebo. The increased risk of developing CHD in women receiving estrogen + progestin is highlighted by the increase adjusted hazard ratio of 1.24 (blue box).



**Figure 1.4**

**Table 1. Coronary Outcomes among Women Randomly Assigned to Estrogen plus Progestin, as Compared with Those Assigned to Placebo.\***

| Variable                          | Estrogen-plus-Progestin Group<br>(N=8506)   | Placebo Group<br>(N=8102) | Adjusted Hazard Ratio | Nominal 95% CI | Adjusted 95% CI |
|-----------------------------------|---------------------------------------------|---------------------------|-----------------------|----------------|-----------------|
| Mean follow-up time (mo)          | 67.8                                        | 66.8                      |                       |                |                 |
|                                   | <i>no. of cases (annualized percentage)</i> |                           |                       |                |                 |
| <b>CHD</b>                        | 188 (0.39)                                  | 147 (0.33)                | <b>1.24</b>           | 1.00–1.54      | 0.97–1.60       |
| Nonfatal MI                       |                                             |                           |                       |                |                 |
| Including silent MI               | 151 (0.31)                                  | 114 (0.25)                | 1.28                  | 1.00–1.63      | 0.96–1.70       |
| Excluding silent MI               | 147 (0.31)                                  | 109 (0.24)                | 1.30                  | 1.01–1.67      | 0.97–1.74       |
| Death due to CHD                  | 39 (0.08)                                   | 34 (0.08)                 | 1.10                  | 0.70–1.75      | 0.65–1.89       |
| CHD, revascularization, or angina | 369 (0.77)                                  | 356 (0.79)                | 1.00                  | 0.86–1.15      | 0.82–1.22       |
| CABG or PTCA                      | 214 (0.45)                                  | 205 (0.45)                | 1.01                  | 0.83–1.22      | 0.77–1.31       |
| Hospitalization for angina        | 172 (0.36)                                  | 195 (0.43)                | 0.86                  | 0.70–1.05      | 0.65–1.13       |
| Confirmed angina                  | 106 (0.22)                                  | 126 (0.28)                | 0.82                  | 0.63–1.06      | 0.57–1.17       |
| Acute coronary syndrome           | 322 (0.67)                                  | 299 (0.66)                | 1.03                  | 0.88–1.21      | 0.83–1.28       |
| Congestive heart failure          | 113 (0.23)                                  | 109 (0.24)                | 0.99                  | 0.76–1.29      | 0.69–1.42       |

Writing Group for the Women's Health Initiative Investigators JAMA, 2002; 288:312-323  
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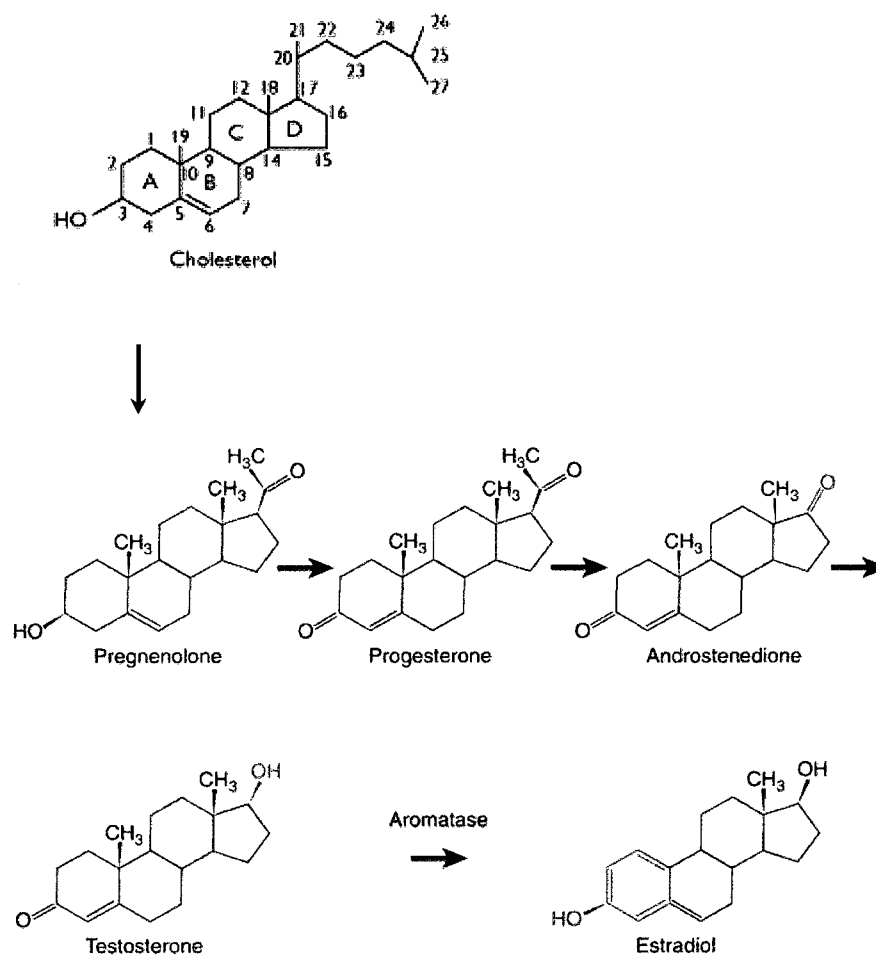
## 1.3 Estrogen and Estrogen Receptors

### 1.3.1 Biological Actions of Estrogen

Estrogen is a sex hormone produced mainly by the ovaries in women throughout the estrous cycle, from menarche to menopause. Synthesis of estrogen from cholesterol results in the formation of the four steroid rings (**Figure 1.5**). In men, estrogen can be generated from aromatase-mediated conversion of testosterone in tissues like the brain, blood vessels and adipose (89). There are three main components of estrogen in women: estradiol, estriol and estrone (89). Of these,  $17\beta$ -estradiol (also referred to as E2) is the main circulating estrogen in cycling women. Levels of estrogen are high during ovulation (600 pg/ml) and peak during pregnancy (20,000 pg/ml). With increasing age, levels of estrogen begin to decline, until menopause, where levels equal those found in men (5-20 pg/ml) (69). Estrogens have many targets in the body, including reproductive tissues like the uterus and breast, as well as non-reproductive tissues like bone and blood vessels (89). Indeed increased cardiovascular disease is not the only threat to post-menopausal women, as lack of estrogen also results in loss of bone mineral density leading to osteoporosis, as well as vasomotor changes that are a major factor in declining quality of life for many women. While certainly the benefits of estrogen replacement were not observed with the clinical trials to date, a better understanding of the diverse and clinically relevant actions of estrogen on the vessel wall is needed. Below is an overview of how estrogen acts through its two major receptors in the cell, to induce

**Figure 1.5: Synthesis of Estrogen.** Using cholesterol as a substrate, estrogen is synthesized with progesterone and testosterone as intermediates.

**Figure 1.5**



Mendelsohn and Karas, Science, 2005; 308: 1583-1587  
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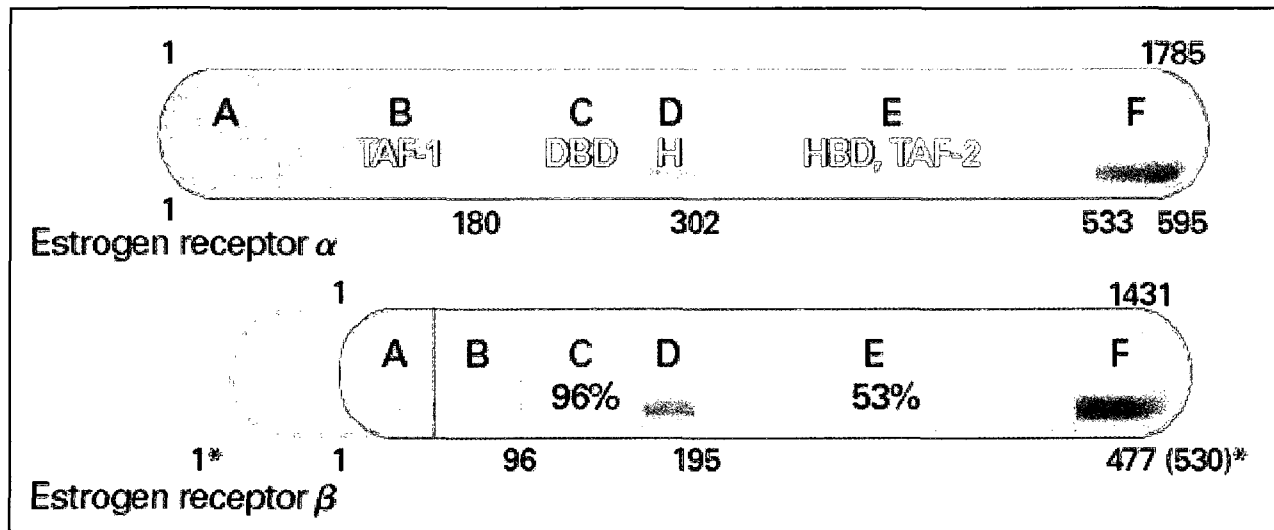
changes in signaling cascades and gene activation that have important consequences in the vasculature.

### **1.3.2 Estrogen Receptors: ER $\alpha$ and ER $\beta$**

There are two primary estrogen receptors: ER $\alpha$  and ER $\beta$ . The discovery of ER $\beta$  in 1996 widened our understanding of how cells can respond differently to estrogen depending on which receptor is engaged (65). ER $\alpha$  and ER $\beta$  are the product of two distinct genes: ESR1 and ESR2. Structurally, ER $\alpha$  and ER $\beta$  share considerable homology (**Figure 1.6**). Like many nuclear receptors, the ERs are subdivided into domains: the DNA binding domain (DBD), the ligand/hormone binding domain (HBD), and two transcriptional activation domains: TAF-1 at the N-terminal, which is ligand independent, and TAF-2 at the C-terminal, which is ligand-dependent. Although ER $\alpha$  and ER $\beta$  are highly similar within the DBD (96% identical at the sequence level), there are two notable areas wherein these two receptors differ considerably. First, ER $\beta$  is only 53% identical to ER $\alpha$  within the HBD. This has become extremely important in the design of specific estrogen receptor modulators that can specifically target ER $\beta$  and not ER $\alpha$ , or vice versa (discussed in detail below). Secondly, the TAF-1 domain in the A/B region at the N-terminus shares only 18% amino acid similarity between the two receptors, making this the most unique part of the molecule (62). Like many other nuclear receptors, adaptor molecules can physically associate with the ERs to modulate their function. This occurs primarily in the TAF-1 and TAF-2 domains, the former being ligand independent and the latter ligand dependent (62). Given that the TAF-1 domain is the most unique portion of ER $\beta$  compared to ER $\alpha$ , it is one of the main reasons that the functions of these two receptors can be so divergent. And as will be discussed

**Figure 1.6: ER $\alpha$  and ER $\beta$  are the Two Primary Estrogen Receptors.** ER $\alpha$  and ER $\beta$  have the classic nuclear hormone receptor structure, with two transactivation function domains (TAF-1 and TAF-2), a DNA binding domain (DBD) and a hormone/ligand binding domain (HBD). They share 96% sequence identity in the DBD, but only 53% and 20% sequence similarity in the HBD and TAF-1 domains, respectively.

**Figure 1.6**



Mendelsohn and Karas, N Engl J Med, 1999; 340 (23):1801-1811.  
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throughout this thesis, it is primarily through this difference in ER $\beta$  that it is thought to be an important molecular target for the treatment and prevention of cardiovascular disease.

### 1.3.3 Estrogen Receptor Ligands

Estrogen is capable of binding both ER $\alpha$  and ER $\beta$  in the form of homo- or heterodimers (reviewed in 4). Although the two receptors differ considerably in terms of sequence and overall structure of their HBD (as discussed above), estrogen appears to bind both receptors with equal affinity (49). There are small but important differences in the three-dimensional structure of this domain: due to a slight variation in amino acid sequence, the pocket where ligand can sit within ER $\beta$  is somewhat larger and more flexible and thus can accommodate larger molecules. This has allowed for the design of highly selective estrogen receptor modulators (SERMs) that make use of this difference in structure between ER $\alpha$  and ER $\beta$ . For example, diarylpropionitrile (DPN) has >50-fold binding affinity for ER $\beta$  over ER $\alpha$ , and has >40-fold transactivation potential for ER $\beta$  over ER $\alpha$  (as measured by increase in gene activation) (**Figure 1.7**) (30). Similarly, an ER $\alpha$  selective ligand propylpyrazole-(1,3,5-triyl)trisphenol (PPT) has >400 fold binding affinity and >20-fold transactivation potential for ER $\alpha$  over ER $\beta$  (30). The specificity and cross-reactivity of the various SERMs is of particular interest for therapeutic purposes, because of the difference in tissue distribution and activity of ER $\alpha$  versus ER $\beta$ . For example, stimulation of ER $\alpha$  is necessary to prevent bone loss and weight gain, and is generally targeted to prevent the vasomotor changes associated with menopause (7). However, due to its high expression in the uterus and breast, an undesirable side-effect of this activation via ER $\alpha$  is a hypertrophic uterus response and increased risk for breast tumors, as was demonstrated in a variety of estrogen



**Figure 1.7: Selective Estrogen Receptor Modulators (SERMs) and their binding and transactivation potencies.** Selective ER $\alpha$  and ER $\beta$  agonists are compared to estrogen (E2) in terms of binding affinity (using receptor binding assays) and transactivation potencies (using estrogen response element reporter assays). E2 is used as reference and is set at 100%.

**Figure 1.7**

| Compounds        |                                      |                    | Binding Affinity <sup>2</sup> |             |                | Transactivation Potency <sup>3</sup> |             |                |
|------------------|--------------------------------------|--------------------|-------------------------------|-------------|----------------|--------------------------------------|-------------|----------------|
| Name             | Property                             | Source             | ER $\beta$                    | ER $\alpha$ | $\beta/\alpha$ | ER $\beta$                           | ER $\alpha$ | $\beta/\alpha$ |
| E2 <sup>1</sup>  | ER agonists                          | Sigma              | 100                           | 100         | 1              | 100                                  | 100         | 1              |
| D1               | ER $\beta$ selective agonist         | Durst <sup>4</sup> | 21.5                          | 1.5         | 14             | 162                                  | 4.6         | 35             |
| D32              | Methy derivative of D1               | Durst <sup>4</sup> | 49                            | 4.3         | 11             | 149                                  | -10         | N/A            |
| 8 $\beta$ -VE2   | Highly selective ER $\beta$ agonist  | Bayer              | 22                            | 0.35        | 63             | 34                                   | 0.11        | 310            |
| DPN              | Highly selective ER $\beta$ agonist  | Tocris             | 6.6                           | 0.12        | 55             | 3                                    | 0.063       | 48             |
| 16 $\alpha$ -LE2 | Highly selective ER $\alpha$ agonist | Bayer              | 0.09                          | 14.6        | 0.006          | 0.07                                 | 18.9        | 0.004          |
| PPT              | Highly selective ER $\alpha$ agonist | Tocris             | 0.12                          | 10          | 0.01           | 0                                    | 20          | 0              |
| Raloxifene       | SERM                                 | Lilly              | 0.54                          | 7.8         | 0.07           | N/A                                  |             |                |

<sup>1</sup> E2=17 $\beta$ -estradiol

<sup>2, 3</sup> The binding affinity and transactivation of E2 are arbitrarily decided as 100; those of other SERMs listed in the table are all relative to E2

<sup>4</sup> Asim et al, Bioorg Med Chem Lett 19:1250-1253.2009

replacement models of atherosclerosis (85). However, as a result of its varied tissue distribution and ability to antagonize the activity of ER $\alpha$ , the unwanted uterine and breast response can be avoided if ER $\beta$  is chosen as the specific target. Recently, the generation of highly specific ER $\beta$  agonists has generated considerable excitement over the possibility of using these compounds for the treatment of a variety of hormone-deficient conditions while maintaining safety by avoiding reproductive malignancies (**Figure 1.7**, Durst compounds) (7). The future of HRT will rely on the ligand affinity difference between ER $\alpha$  and ER $\beta$  with selectivity for ER $\beta$  to avoid unwanted effects on reproductive organs, especially for the treatment of cardiovascular disease.

### **1.3.4 Cellular Mechanisms of Estrogen Action**

#### *1.3.4.1 Nuclear genomic effects of estrogen*

Estrogen receptors are traditionally considered nuclear hormone receptors, capable of binding DNA upstream of target genes and modulating their expression. In the majority of cell types, ER $\alpha$  and ER $\beta$  expression is predominantly found in the nucleus, where upon ligand binding, a conformational change occurs, receptors form dimers and bind target genes (87). ERs possess a DNA binding domain which, as discussed above, shares considerable homology between ER $\alpha$  and ER $\beta$ . This domain recognizes a particular sequence, termed an estrogen response element (ERE), in the promoter region of certain genes (10,116). EREs consist of a consensus sequence of 13 nucleotides, with the palindromic arrangement 5'-GGTCAnnnTGACC-3' (where n=any nucleotide) (9). This ERE consensus sequence has been described in a number of promoters upstream of a variety of target genes (10). Of

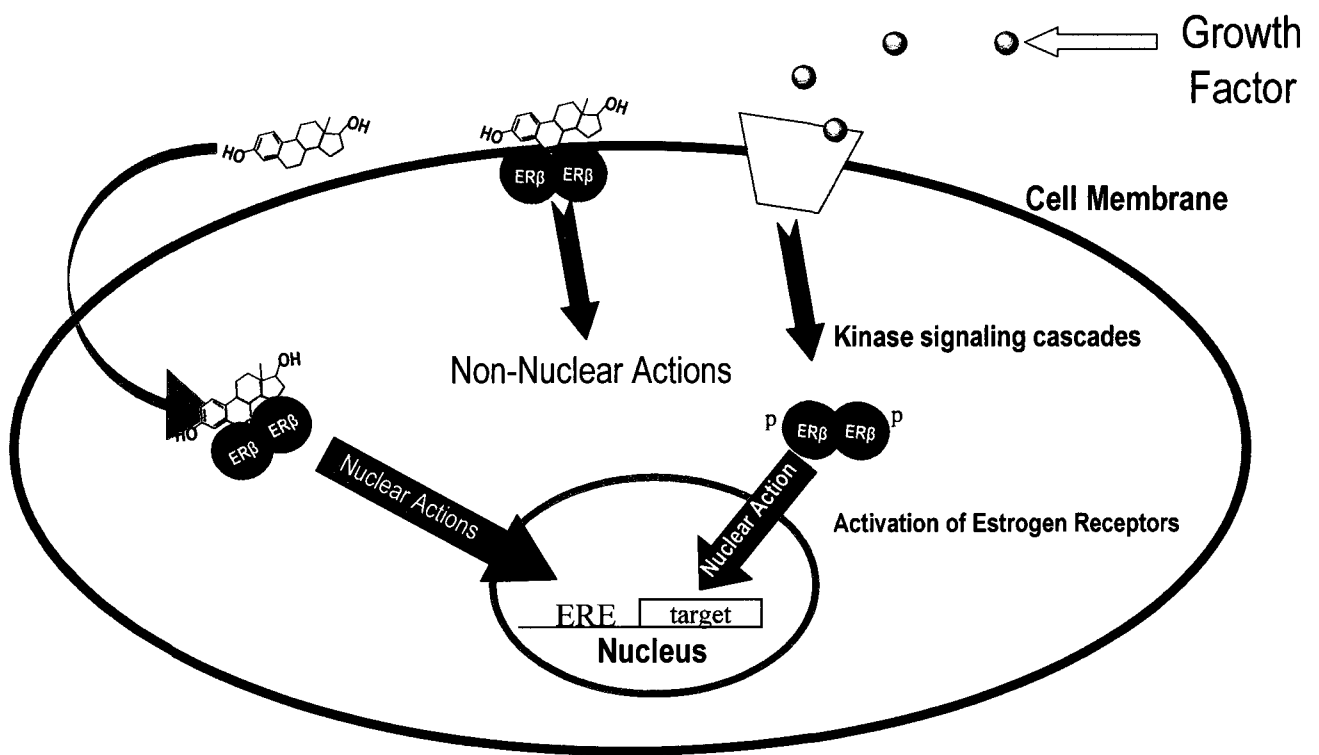
importance to the vascular system, genes that are under the control of an ERE include vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), interleukin-6 (IL-6), vascular cell adhesion molecules (VCAM-1), lipoproteins A and E, and eNOS (reviewed in 80). Estrogen can also influence gene expression in target genes that do not contain an ERE, but rather contain other transcriptional activation sites (like Ap-1 and Sp-1) with the ERs binding c-Fos and c-Jun to regulate transcription at these sites (76,124). Upon ligand binding, receptors bind DNA, and depending on the presence or absence of co-regulatory molecules (discussed below), this will result in the up- or down-regulation of transcription (**Figure 1.8**). Although it was once believed that only cells of the reproductive organs (such as uterus, ovary and prostate) were regulated at the transcriptional level by hormones, it is now appreciated that many non-reproductive tissues (such as the cardiovascular system) not only express functional estrogen receptors, but are directly controlled by estrogen receptor alteration in gene expression profile (79).

#### *1.3.4.2 Non-genomic effects of estrogen*

In addition to these so-called traditional nuclear effects, estrogen can act outside the nucleus to alter cell function. The most compelling argument for this comes from the rapid activation of eNOS in endothelial cells and subsequent vasodilation that occurs in the absence of transcriptional signaling (39). This rapid effect implies that this change occurs in the absence of a change in gene expression, and that perhaps the nuclear activity of ERs is not required. Indeed, estrogen receptors can be found outside the nucleus, in the cytoplasm and at the cell membrane (**Figure 1.9**). These receptors participate in G-protein coupled signaling, regulate ion channel activity, and activate tyrosine kinases (reviewed in 4 and

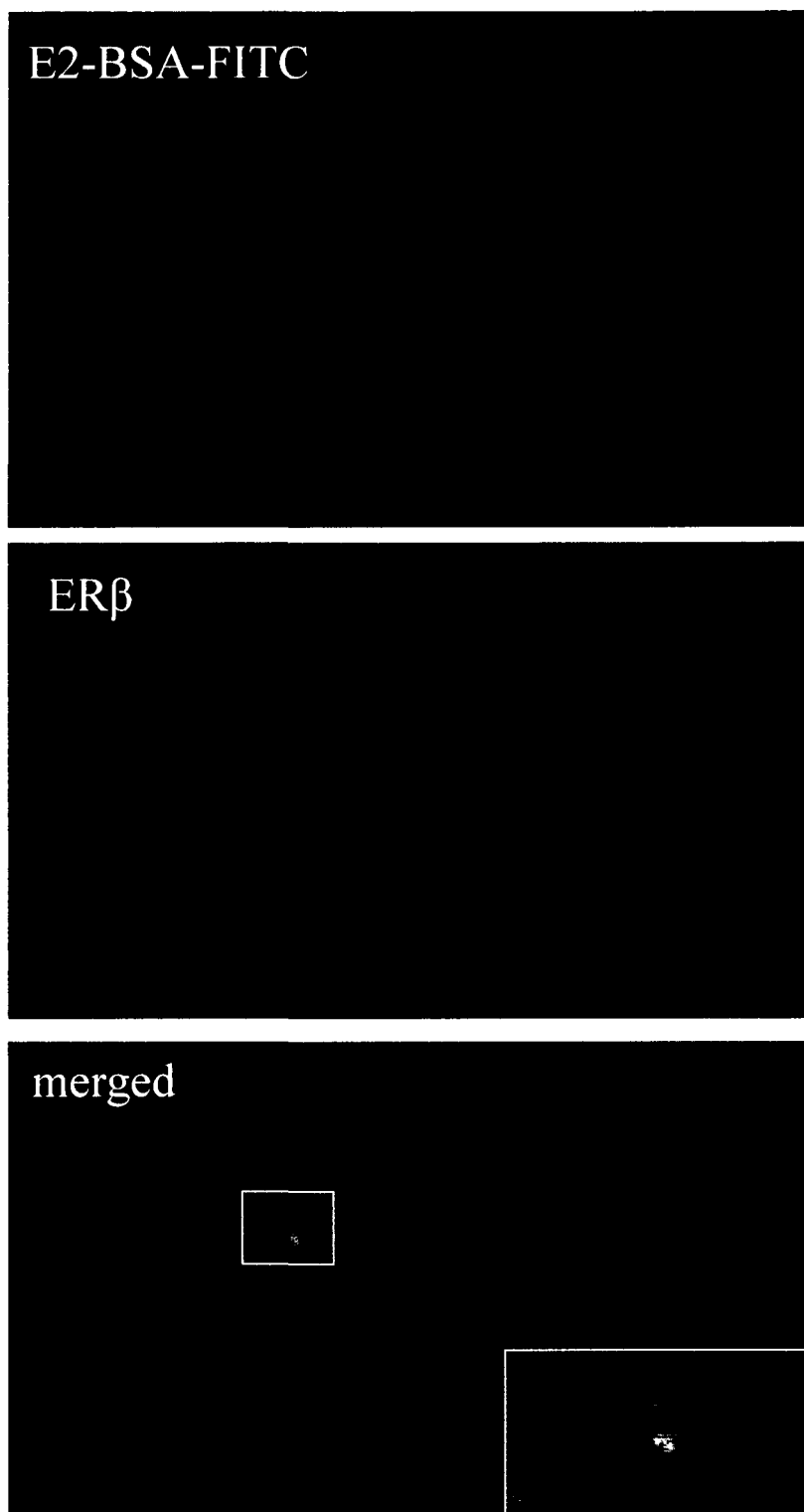
**Figure 1.8: Nuclear and Non-nuclear Actions of Estrogen.** Estrogen can enter the cell by diffusing passively through the cell membrane, where it can bind either ER $\alpha$  or ER $\beta$  in the form of homo- or heterodimers. The receptors can then bind estrogen response elements (ERE) upstream of target genes to activate or repress transcription.

**Figure 1.8**



**Figure 1.9: Estrogen Receptors can be found at the cell membrane.** Estrogen conjugated to BSA and labeled green with FITC (E2-BSA-FITC, top panel) co-localizes in macrophages with ER $\beta$  (blue, middle panel) at the cell membrane (merged, bottom panel)

**Figure 1.9**





35). It is through membrane ERs that estrogen is thought to mediate some protection from vascular injury, via the activation of MAPK pathways and protection of endothelial actin stress fibres during injury (114). As well, ER $\beta$  has been shown to be present in mitochondria (130), and estrogen can increase mitochondrial energy production while decreasing reactive oxygen species production (122). Although the exact role of ER $\beta$  in the mitochondria is unclear, it is an example of how the paradigm of traditional nuclear action of ERs is shifting. The participation of these ERs in important cellular functions within the vasculature is not yet fully understood, and there is still much insight to be gained into additional mechanisms of this non-genomic ER action.

### **1.3.5 Co-regulatory molecules**

Recent discoveries of novel proteins that associate with nuclear receptors have brought about a new paradigm for hormone action. Given that these ER associated or co-regulatory proteins modulate the function of the ERs, the importance of the physiological and pathophysiological response to hormones may reside with these molecules rather than with the receptors themselves. Approximately 300 nuclear receptor (NR) associated proteins are known, primarily discovered using yeast two hybrid screens that employ the NR as “bait” (91,115). While not all NR associated proteins have defined regulatory roles, many act as either activators or repressors of receptor transcriptional activity. Typically, these proteins do not bind DNA themselves, but instead facilitate the interaction of the hormone receptors with DNA and other structural proteins, either serving to facilitate (activators) or hinder (repressors) the activation of transcription (91). While there are some co-regulatory molecules that share common features (such as amino acid sequence or structural

similarities), many receptor co-regulators are often well-characterized mediators of other signaling pathways (e.g. E6-AP, known as a ubiquitin ligase, can activate ERs) (36). Regarding their mechanism of action, data are emerging to suggest an important role of NR associated proteins in various disease processes, by mediating the biological effects of steroid hormones. For example, in women, steroid receptor co-activators of the p160 family (well-known activators of ER transcription) are expressed in the normal endometrium during the menstrual cycle but over-expressed in women with polycystic ovary disease and appear to contribute to poor reproductive function, endometrial hyperplasia, and cancer (41). It was recently demonstrated that another activator of ER transcription E6-AP protein expression is down-regulated in invasive breast and prostate carcinomas, and correlated with over-expression of ER $\alpha$  and androgen receptor (AR), respectively (36). In the vessel wall, expression of steroid receptor coactivator-1 (SRC-1) or SRC-3 is required for the protection from vascular injury via estrogen (131,132). These examples highlight the importance of examining proteins that regulate ER $\alpha$  and ER $\beta$  in order to better understand how these proteins might participate in cardiovascular disease progression and protection.

In the subsequent chapters of this thesis, new data will be presented to suggest that novel ER co-regulatory molecules have important functions in the development of atherosclerosis and that these proteins rather than the receptors themselves may be the target of future therapeutic intervention.

## **1.4 Estrogen Action in the Vasculature**

In the healthy vessel wall, both ECs and SMCs express functional estrogen receptors ER $\alpha$  and ER $\beta$  (80). Macrophages also express functional ERs, and thus may be a target for therapeutic hormone intervention within atherosclerotic plaques (17). Each cell type responds differently to stimulation with estrogen under normal and pathological conditions, adding a layer of complexity when attempting to understand the vascular response to this hormone, both in normal and pathological settings.

### **1.4.1 Estrogenic Effects on Vascular Cells**

#### *1.4.1.1 The Endothelium*

Under normal conditions in the vessel wall, ECs are aligned on the luminal surface of the vessel wall and are involved in maintaining vessel wall homeostasis. These cells are joined together by tight junctions and provide a barrier between circulating blood cells and the underlying tissues, maintaining vessel wall tone and regulating the inflammatory response in the vasculature (1). A healthy endothelium is crucial to the maintenance of a healthy vessel wall, and disruption of this barrier leads to pathological complications like atherosclerosis. ECs express both ER $\alpha$  and ER $\beta$ , both in the nucleus and at the cell membrane (63). Estrogen has systemic effects on the endothelium that are thought to contribute to its anti-atherogenic effects: firstly, estrogen regulates vasodilation by activating the nitric oxide (NO) pathway. In the short-term, estrogen binds the estrogen receptors and causes the rapid release of NO from vesicles, downstream of PI3K and Akt (47). This results in the activation of ion channels and subsequent relaxation of underlying smooth muscle cells, which ultimately

leads to vasodilation (reviewed in 88). Similarly, long-term estrogen exposure results in the increased transcription of endothelial nitric oxide synthase (eNOS), an enzyme involved in the production of NO, ensuring long-term availability of NO (59). These combined estrogen-mediated vasodilatory and anti-inflammatory effects of NO on the endothelium are believed to play an important role in maintaining vessel wall homeostasis, and are thought to be one explanation for the lower blood pressure and subsequent lower degree of atherosclerosis in pre-menopausal women compared to age-matched men (26,105).

Secondly, estrogen has positive effects on overall cholesterol metabolism that aids in the maintenance of a healthy endothelium, both by decreasing the level of oxidized LDL and by affecting the hepatic production of lipoproteins (reviewed in (64). As previously discussed, oxidized LDL is a major contributor to the development of cardiovascular disease, thus lower levels are beneficial. Moreover, oxidized LDL can decrease the “bioactivity” of NO, which as discussed above, can be harmful to the endothelium (99). Estrogen also increases the levels of circulating HDL, which can prevent the available LDL from undergoing oxidation (109). This in turn enhances overall endothelial function due to the reduction in harmful oxidized LDL and improved metabolism of circulating cholesterol. Reducing harmful cholesterol deposits in turn reduces foam cell formation in both SMCs and macrophages, contributing overall to a reduction in lesion development.

In addition to these systemic effects, estrogen has been shown to alter the proliferation and migration of ECs in culture: 17 $\beta$ -estradiol can increase the number of porcine ECs *in vitro*, and can enhance the formation of tubular EC networks and increase their chemotactic activity (37,84). Together, these *in vitro* effects are consistent with the hypothesis that *in vivo* estrogen facilitates the maintenance of a healthy endothelium and promotes re-

endothelialization- processes which are both dependent upon EC proliferation and migration. Indeed, studies have shown that in the absence of a healthy, intact endothelial layer, any beneficial effects of estrogen on atherosclerosis are abolished (54).

#### *1.4.1.2 Smooth Muscle Cells*

SMCs that line the medial layer of the vessel wall directly below the endothelium are involved in both maintenance of normal contraction of the blood vessel and pathological processes that underlie diseases like atherosclerosis. Under normal conditions, SMCs maintain a quiescent phenotype, largely regulated by a healthy endothelium (108). However, during disease processes, often as a result of a compromised endothelium, SMCs begin to proliferate, migrate and secrete proteases and extracellular matrix proteins that contribute to lesion development (108). Estrogen appears to be capable of attenuating the activation of these SMCs. For example, studies have demonstrated that 17 $\beta$ -estradiol has an inhibitory effect on SMC proliferation both *in vivo* and *in vitro*, as demonstrated by a decrease in neointima formation and cell replication, respectively (106). Additionally, estrogen has been shown to reduce growth-factor induced SMC migration and MAP kinase activation (37). Therefore estrogen appears to be capable of reducing the disease-promoting actions of smooth muscle cells. Interestingly, in the setting of high levels of oxLDL, differential methylation of the estrogen receptor  $\alpha$  promoter can occur, leading to loss of ER expression and therefore loss of anti-proliferative effects of estrogen on SMCs in atherosclerosis (57).

#### *1.4.1.3 Macrophages and Foam Cells*

Macrophages migrating into the vessel wall in response to either oxLDL and/or an activated endothelium are the primary perpetrator of atherogenesis, activating the inflammatory cascade with subsequent recruitment of additional inflammatory cells. However, it is believed that estrogen can abrogate the inflammatory response that typically accompanies macrophage infiltration into the vessel wall (44). Activation of the NF $\kappa$ B pathway is central to the production of many inflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ . Estrogen can inhibit the nuclear translocation and subsequent gene activation by NF $\kappa$ B (38). Estrogen also influences the expression of chemoattractant proteins by macrophages. For example, treatment of ovariectomized mice with estrogen resulted in decreased expression of monocyte chemoattractant protein-1 (MCP-1), a key player in promotion of inflammatory atherosclerosis (111). Estrogen can also reduce the production of TNF $\alpha$ , a key inflammatory mediator, possibly via ER $\beta$  (3,117). Moreover, treatment of macrophages with estrogen resulted in increased cholesterol efflux from macrophages, resulting in reduced accumulation of cholesterol and reduced foam cell formation (86). Taken together, these data suggest that estrogen influences macrophages to adopt a less inflammatory phenotype with reduced foam cell formation, resulting in beneficial effects on the surrounding vascular cells and reduced plaque formation.

#### **1.4.2 Estrogen and Animal Models of Atherosclerosis**

In order to directly address the role of estrogen in protection from atherosclerosis, a number of animal models of atherosclerosis combined with estrogen replacement have been conducted. The first study demonstrating a protective role for estrogens employed female

apoE<sup>-/-</sup> mice ovariectomized bilaterally at 4 weeks of age (13). Three weeks later, mice either received low-, medium- or high-dose estrogen pellets (6, 12 or 28 µg/day, respectively) subcutaneously before the commencement of either a high-fat western diet or a control chow diet for three months. The doses of estrogen were expected to achieve serum levels in the range of 100pg/ml to 900pg/ml, with the lowest dose comparable to non-ovariectomized mice at peak estrus (28). All three doses of estrogen reduced lesion size in both the aortic sinus and the descending aorta, with reductions ranging from 40 to 70% in chow fed animals, and 40-83% reductions in high-fat fed animals (13). Similarly, another study showed that treatment of ovariectomized apoE<sup>-/-</sup> mice with lower doses of estrogen following ovariectomy (0.17-8.3 µg/day) reduced aortic sinus atherosclerotic lesion size by 30-75% in a dose-dependent manner (29). In each of these studies, total circulating cholesterol levels were reduced following estrogen replacement by as much as 50%; however, Bourassa et al. demonstrate that plasma cholesterol levels only weakly associate with lesion area, suggesting that estrogen has protective effects that extend beyond those provided by reducing serum cholesterol (13). And finally, in a similar mouse model of atherosclerosis, LDLR<sup>-/-</sup> mice ovariectomized and treated with 6.7 µg/day estrogen demonstrated dramatic 70% reductions in *en face* aortic lesion area compared to non-treated mice after high-fat diet- despite the lack of change in total serum cholesterol in the treated animals (74). These data overwhelmingly suggest that estrogen replacement is highly protective against atherosclerosis, although changes in total circulating cholesterol may only be one mechanism through which estrogen is exerting its beneficial effects. However in each of these studies, replacement of estrogen following ovariectomy caused dramatic increases in uterine weight, as high as two-fold compared to placebo-treated animals. This effect was shown to be mediated by ERα, as mice deficient in ERα did not have appreciable changes in

their uterine weight following estrogen replacement, despite plasma estrogen concentrations greater than 100 pg/ml (51). Therefore, although replacing estrogen in mouse models of atherosclerosis was able to protect from disease, unwanted side effects like increases in uterine weight were unavoidable.

### **1.4.3 ER $\alpha$ versus ER $\beta$ in the Vessel Wall**

Given that both ECs and SMCs express both receptors, it would appear at first glance that ER $\alpha$  and ER $\beta$  may have redundant functions; however, studies have shown that this is not the case. Initial studies examining the role for ER $\alpha$  and ER $\beta$  in the vasculature employed both *in vitro* and *in vivo* models of vascular injury, measuring the contribution of each receptor to SMC and EC proliferation and migration. For example, using siRNA to specifically eliminate expression of either ER $\alpha$  or ER $\beta$  in vascular cells, Geraldles *et al.* showed that each ER differs in its specific contribution to activation of ECs and SMCs and that one receptor may require the presence of the other to function normally (37). They demonstrate that by removing ER $\alpha$  from porcine vascular cells, there is no effect on aortic SMC proliferation and migration, but a significant effect on aortic EC proliferation and migration. In contrast, the removal of ER $\beta$  from these cell types results in changes to SMC proliferation and migration, yet ECs remain unchanged. Indeed when the first knock-out mouse model for ER $\alpha$  was described, using a model of vascular injury it was concluded that ER $\beta$  must be the principal receptor mediating the protective effects of estrogen because estrogen continued to have protective effects in the vasculature despite the absence of this “primary” receptor, (60). However, similar experiments from ER $\beta$  knock-out mice showed the same cardioprotective effects (61). Initial reports of the deletion of ER $\alpha$  in a mouse



model of atherosclerosis suggested that ER $\alpha$  must mediate the majority of the anti-atherogenic properties of estrogen, as mice deficient in ER $\alpha$  showed no reduction in lesion size upon treatment with estrogen compared to those with intact ER $\alpha$  (51). However, the deletion of ER $\alpha$  in these models was incomplete, and splice variants of ER $\alpha$  remained active in these mice (23). Consequently, any conclusions reached regarding the role for ER $\alpha$  or ER $\beta$  using this incomplete knock-out are invalid. Unfortunately, although a new ER $\alpha$  knock-out mouse was generated, these initial studies confounded our understanding of how ER $\alpha$  and ER $\beta$  function in the vessel wall. Therefore, based on the conclusions from animal models of both vascular injury and atherosclerosis, the understanding of the role for ER $\alpha$  and ER $\beta$  in the vessel wall remains incomplete. Perhaps the major contribution to our understanding of estrogen action in the vessel wall from these models is to underscore how complicated the interplay between these two receptors can be. The unambiguous role for ER $\alpha$  and ER $\beta$  in the cardiovascular system remains to be elucidated, and further study into the specific contribution of each of these receptors is warranted.

In summary, evidence suggests that estrogen acts on the vasculature by promoting endothelial health and function, reducing the pathological SMC phenotype, reducing circulating cholesterol and reducing inflammation in macrophages in the vessel wall. This begs the question: why did hormone replacement therapy fail to show any cardiovascular benefit? The mechanism of estrogen action in the vessel wall is complex and multi-faceted. The work brought forth in this doctoral thesis will attempt to reconcile the controversies surrounding the cardiovascular benefits of HRT and will probe deeper into the molecular and

cellular understanding of estrogen and its receptors as it relates to physiological and pathological outcomes.

#### **1.4.4 The Importance of ER $\beta$ in Cardiovascular Disease**

Until about a decade ago, ER $\alpha$  was thought to be the only estrogen receptor, and it was only in 1996 when ER $\beta$  was discovered that the entire ER story had to be re-thought (65). Indeed the confusion regarding the outcomes of the ER $\alpha$  and ER $\beta$  knock-out models of atherosclerosis underscored the need to better understand how each receptor behaves in the vessel wall. Despite this confusion, there is a growing body of both *in vitro* and *in vivo* evidence supporting an important involvement of ER $\beta$  in the development of cardiovascular disease:

- i) ER $\beta$  mRNA and protein are abundantly expressed in human coronary arteries;  
(42,90)
- ii) ER $\beta$  mRNA and protein expression is increased in response to vascular injury;  
(52,68)
- iii) ER $\beta$  is the predominant mRNA transcript in human vascular SMCs in women;  
(50)
- iv) ER $\beta$  expression predominates in the arteries of men with early stages of atherosclerosis, and intimal ER $\beta$  (but not ER $\alpha$ ) expression correlates with coronary calcification and atherosclerosis in pre- and post-menopausal women;  
(70)

- v) Treatment with an ER $\beta$  agonist (biochanin A) resulted in decreased neointima formation and preserved vascular function in a rat wire injury model; (107)
- vi) Recent compelling evidence from our lab demonstrated that treatment of atherosclerosis-prone mice with a highly ER $\beta$ -specific agonist results in a profound reduction in atherosclerotic lesion area; (123).

Given the above evidence in support of ER $\beta$  in modulating CAD, ER $\beta$  is a rational choice for hormonal therapeutic intervention. Moreover, targeting ER $\beta$  instead of ER $\alpha$  in the vessel wall avoids the undesirable side effects of ER $\alpha$  activation in the reproductive system (i.e. growth of uterine and breast tissue). However, even with the mounting evidence supporting a role for ER $\beta$  in atherosclerosis and vascular diseases, at the outset of this doctoral thesis little was known about the unique aspects of ER $\beta$  in the vessel wall. This prompted the investigation into ER $\beta$  and its specific associated proteins. We performed a yeast-two hybrid assay, a useful tool for detecting novel protein interactions. Using the unique A/B region of ER $\beta$  as bait, which shares less than 20% homology with ER $\alpha$ , we screened a human lung fibroblast cell line cDNA library of  $5.6 \times 10^6$  clones and discovered three true positive interactors: heat shock protein 27 (HSP27), non-metastatic protein 23-H2 (NM23-H2) and clone B8 (a protein with a yet undefined function). The main objective of this doctoral thesis was to understand more about ER $\beta$  and the role of these novel associated proteins in the vessel wall. As will be explained in detail in Chapters 1 & 2, as well as in Appendix I, HSP27 and NM23-H2 were characterized and found to have important roles in vascular disease.

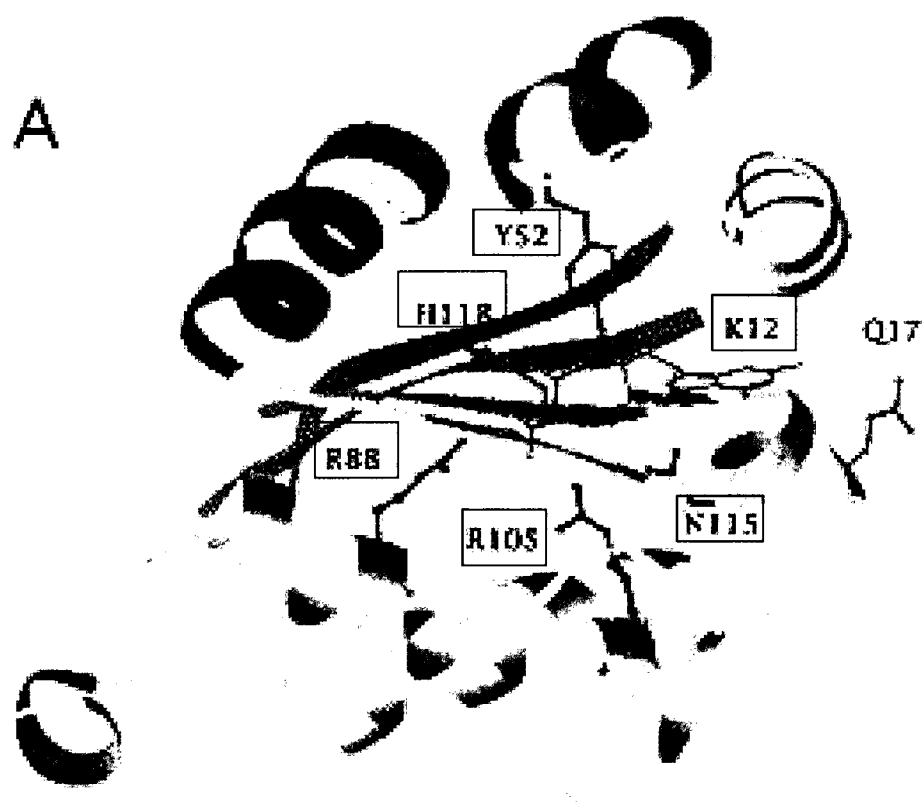
## **1.5 Novel Estrogen Receptor $\beta$ Associated Protein**

### **1.5.1 NM23-H2**

The non-metastatic protein family (NM23) was originally described in the cancer literature (33,45). Conserved in drosophila, yeast, bacteria and mammals, the NM23 genes were first described as nucleoside diphosphate kinases (NDPKs) but have emerged as regulators of a variety of both normal and pathological functions (97). There are eight NM23 family members described to date (45). NM23-H2 is a rather distinct member of this family, being functionally different from its closely-related counterpart, NM23-H1, despite sharing 88% sequence identity (5). Structurally, NM23-H2 contains both DNA binding properties as well as NDP kinase properties, each utilizing different amino acids to function (**Figure 1.10**) (97). This makes NM23-H2 unique, because unlike other members of the NM23 family, NM23-H2 functions not only as an NDP kinase, but also as a potent activator of the c-myc promoter (98). This regulation of c-myc can have profound effects on cell proliferation and differentiation, and is the primary reason this protein is thought to have important functions in many human malignancies (120). As the name suggests, the NM23 proteins are believed to inhibit metastasis of certain cancers due to their effects on cell migration and invasion. For example, by altering expression of proteins associated with cell motility and adhesion (e.g.  $\beta$ 1-integrins present at the cell surface) via alterations in c-myc activation, NM23-H2 can reduce cell migration and adhesion and thus reduce the metastatic potential of tumors (125,134). At cell surface adhesion junctions, NM23-H2 physically associates with the integrin complex of proteins, reducing migration and favouring cell spreading (34). Similarly, NM23-H2 can bind microtubules and interfere with proper cytoskeletal reorganization (72). It is through these protein-protein interactions and alteration of c-myc

**Figure 1.10: Ribbon diagram of NM23-H2.** Residues in BLUE are required for the DNA binding activity, residues in BLACK are required for the NDP kinase activity, and in RED is the active site.

**Figure 1.10**



Postel EH., *Biochemistry*, 2002; 41 (20):6330–6337.  
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activation that NM23-H2 exerts its important biological effects, most of which are independent of its activity as an NDPK (71). Additionally, NM23-H2 can prevent the endocytosis of a G-protein coupled receptor, altering downstream cell signaling events (102). This provides yet another mechanism of cellular regulation via NM23-H2, demonstrating its multi-faceted capabilities.

#### *1.5.1.1 Role of NM23-H2 in the Vessel Wall*

Despite the understanding of NM23-H2 as a kinase or a regulator of c-myc transcription, there was little known about this protein in the vascular system or what role (if any) it plays in vascular diseases. Interestingly, expression analysis revealed that NM23-H2 was the only member of the NM23 family that was ubiquitously expressed in all tissues during mouse embryonic development (2). Perhaps related to its activation of the c-myc promoter, NM23-H2 influences cell differentiation during haematopoiesis, promoting normal erythropoiesis and reduced macrophage development (128). Deletion mutants of NM23-H2 are embryonic lethal (E.H. Postel, personal communication), whereas deletion of NM23-H1 caused no obvious phenotype, highlighting the importance of the H2 isoform during development (12). NM23 is involved in angiogenesis and endothelial cell differentiation *in vitro*, but there are no data to suggest NM23-H2 is expressed in the vascular system (135). As will be highlighted in Manuscripts #1 and #2, we discovered that NM23-H2 is indeed expressed in vascular cells both *in vitro* and *in vivo*, and that this might have important functions regulating estrogen signaling that are lost with the progression of atherosclerosis.

### 1.5.2 HSP27

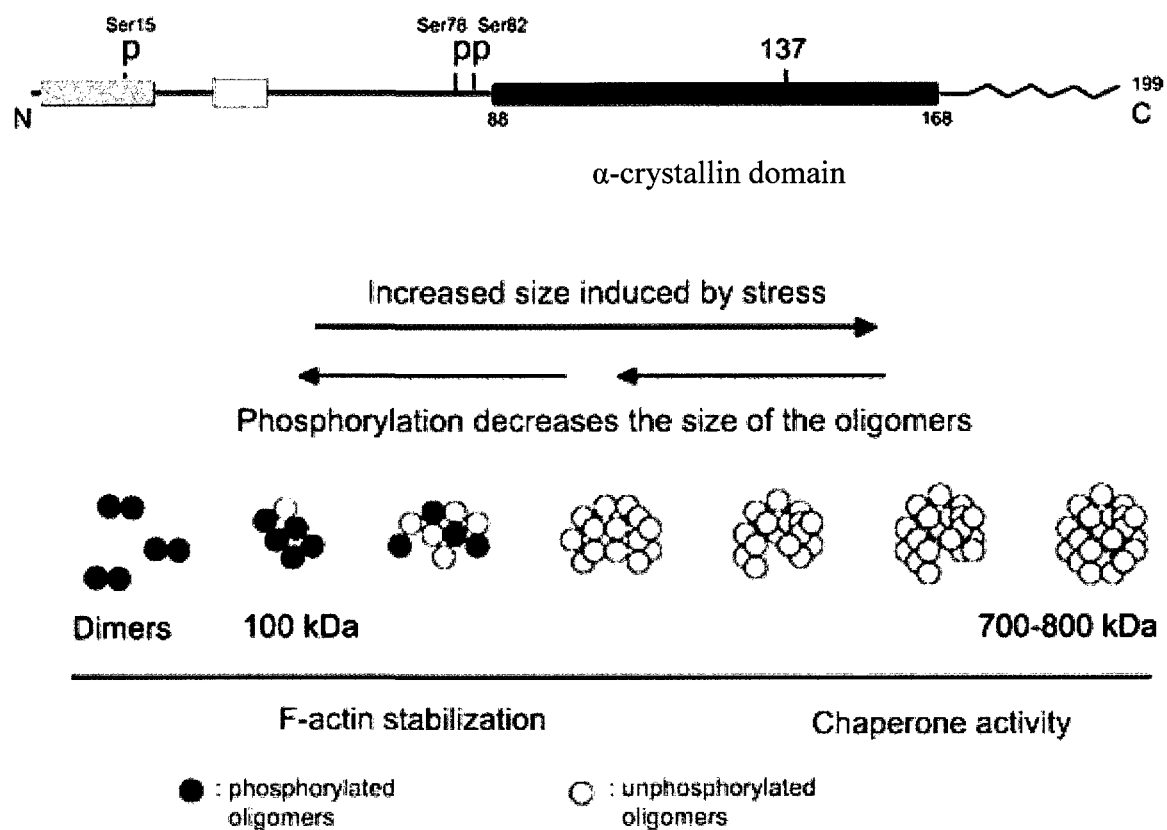
The second ER $\beta$  associated protein that we studied was heat shock protein 27 (HSP27). As a member of the small heat shock protein (sHSP) family, HSP27 is classified as a molecular chaperone that can be upregulated to protect the cell from the sequelae of stressful insults. Due to its constitutive expression in a wide variety of tissues, HSP27 does not require *de novo* protein synthesis and thus can act rapidly in times of stress (6). Although a relatively small (27 kDa) protein, HSP27 can form large oligomers ranging from 50 kDa to greater than 700 kDa in size (**Figure 1.11**) (40,67). HSP27 can be phosphorylated by p38 MAPK primarily at serine residues 15, 78 and 82 (55). Upon phosphorylation, HSP27 multimers undergo dissociation to form smaller dimers, which are thought to be the form in which HSP27 exerts most of its protective effects (77,113). The HSP27 gene is under the control of the heat shock element (HSE) in its promoter region, to which heat shock factor-1 (HSF-1) can bind and activate to upregulate HSP27 in conditions of stress (16,48). HSP27 differs from the other traditional heat shock proteins (e.g. HSP60, HSP90) in that it does not participate in chaperone activity that accompanies routine protein synthesis; instead, HSP27 chaperones specific proteins during distinct biological processes, often preventing proteins from aggregating during heat shock (46,67). Indeed mutations that alter the chaperone activity of HSP27 in mice have deleterious consequences in the response to oxidative stress and induce cardiomyopathy (101). HSP27 is well-known for its role as an actin binding protein, protecting the cytoskeleton from dissociation during times of stress. This occurs through the phosphorylation of HSP27 oligomers that dissociate into smaller dimers to cap the actin stress fibers (**Figure 1.11**) (11). HSP27 is a very potent anti-apoptotic protein by virtue of its ability to prevent the release of cytochrome c and Smac from mitochondria and



**Figure 1.11: Structural organization and oligomerization of HSP27.** HSP27 is a 199 amino acid protein with a conserved  $\alpha$ -crystallin domain and phosphorylated serine residues. HSP27 can exist as large oligomers, which dissociate upon phosphorylation.

**Figure 1.11**

**Hsp27 (HspB1)**



Arrigo et al., FEBS Lett. 2007;581(19):3665-74.  
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prevent the induction of the downstream caspase cascade (21,56,78,95). Similarly, HSP27 can act downstream of mitochondria by physically associating with cytochrome c to prevent apoptosome formation (15). In the context of inflammation, HSP27 can prevent some forms of inflammation by decreasing IL-6 and ICAM-1, possibly through its interaction with the NFκB pathway (93). Overall, HSP27 is regarded as an anti-inflammatory protein, capable of protecting cells and tissue from the threat of pro-inflammatory or cell death-inducing stimuli (e.g. by inducing the expression of IL-10, a potent anti-inflammatory cytokine) (25). *In vivo*, HSP27 has been shown to offer protection from a variety of forms of experimental injury (e.g. motor neuron injury, gastric mucosal injury, and ischemia/reperfusion injury) (27,53,112).

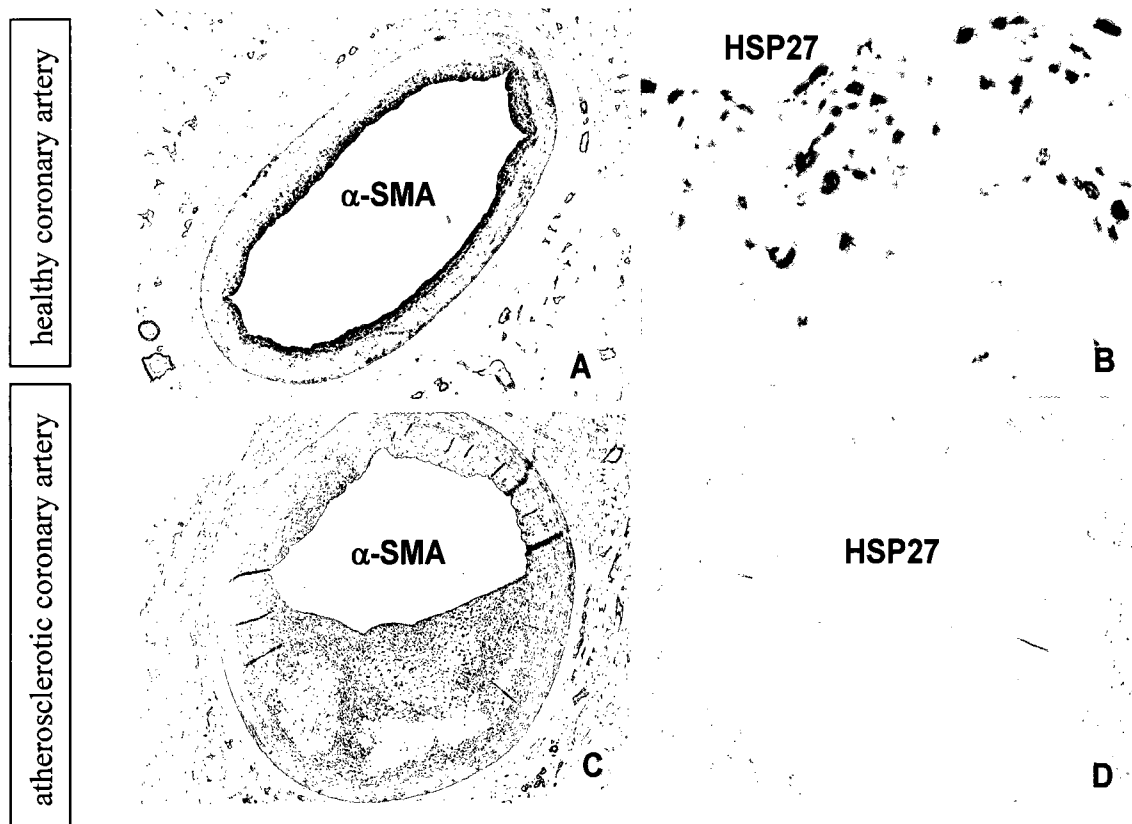
#### *1.5.2.1 Role of HSP27 in the Vessel Wall*

HSP27 protein is expressed by endothelial cells and smooth muscle cells within the vessel wall (82). Compelling human data implicating HSP27 as an important protein with a role in cardiovascular disease development has recently emerged. Two unbiased proteomic screens discovered decreased HSP27 levels secreted from human carotid endarterectomy plaques *vs* normal (disease free) arteries (75,94). Similarly, preserved HSP27 expression in cardiac biopsy specimens correlates with freedom from vascular disease (cardiac allograft vasculopathy) in transplanted hearts (24). We also demonstrated that in arteries containing advanced atheromas, expression of HSP27 is decreased compared to normal healthy arteries (see **Figure 1.12**, and Appendix I). Likewise, we observed that young mice with disease-free arteries have high levels of HSP25 (the mouse homolog of HSP27) while older mice with advanced atherosclerotic lesions in their aortas no longer express HSP25 within the

**Figure 1.12: HSP27 expression is diminished as atherosclerosis progresses.**

Photomicrographs of cross-section of a benign intimal thickening in the coronary artery of a young individual free of atherosclerosis (top row) and with an advanced atheroma with necrotic cholesterol-laden intimal core (bottom row), immunolabeled for  $\alpha$ -smooth muscle actin (A & C; magnification 100x) and HSP27 (B & D; magnification 1000x and 100x respectively).

**Figure 1.12**



Miller et al., Arterioscler Thromb Vasc Biol., 2005, 25(3):e10-4.  
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vessel wall (unpublished observations). Together with other laboratories we noted that HSP27 levels in the serum of individuals suffering from CAD are lower than serum levels of healthy individuals- paralleling the decrease in HSP27 expression observed in the diseased vessel wall (18). These data suggest that HSP27 expression can be protective in the vessel wall, as loss of expression correlates with vascular disease. Interestingly, induction of HSP27 expression by pre-treatment with herbimycin A (a non-specific inducer of heat shock factors) or heat pre-treatment itself dramatically reduces neointimal formation after carotid artery balloon injury relative to untreated controls (22). Independent from its role as an ER $\beta$  associated protein, HSP27 appears to have novel and important roles to play in the vessel wall, implying that strategies aimed at increasing HSP27 expression may be beneficial for maintaining vessel wall homeostasis. As will be discussed in detail in Chapter 4 and Chapter 5, we directly addressed the question of the role of HSP27 in atherosclerosis, and show for the first time that HSP27 is indeed an atheroprotective protein, with estrogen being required for this effect.

## **1.6 Summary**

In summary, while estrogen appears to partially provide the vessel wall with protection, it is clear that these mechanisms are poorly understood- possibly being an explanation for the surprising negative conclusions of the HRT trials. While there are many factors that contribute to how the vessel wall responds to estrogen, including which ligand is present, which cell type is responding and which co-factors are present, the specific contribution of ER $\alpha$  and ER $\beta$  in the vasculature remains unclear. Estrogen can be a double-edged sword,

being both protective in the setting of atherosclerosis but dangerous to reproductive tissues. As we understand more about ERs in general and ER $\beta$  in particular, we will come to better appreciate the mechanisms of estrogen action in the cardiovascular system and how they are distinct from those in other tissues, so that the therapeutic potential of estrogen can be fully realized. The global objective of this doctoral thesis is to understand more about the molecular mediators of estrogen action in the vessel wall by gaining insight into ER $\beta$  action and the action of its cofactors (e.g. NM23-H2 and HSP27). This will help identify how ER $\beta$  may serve as a novel target for the prevention and treatment of cardiovascular disease, ultimately leading to the tailoring of specific hormone therapy for cardiovascular benefit in both women and men.

## **1.7 Hypothesis**

I hypothesize that estrogen receptor  $\beta$  plays an important role in the development and progression of cardiovascular disease, and through its interaction with specific co-regulatory molecules, can modulate the function of estrogen in the vessel wall.

## **1.8 Research Objectives**

In order to test the hypothesis that ER $\beta$  plays an important role in cardiovascular disease, there are three overall specific aims:

- (1) to characterize the association of ER $\beta$  with novel co-regulatory proteins  
NM23-H2 and HSP27
- (2) to elucidate the role of HSP27 in atherosclerosis
- (3) to determine if the atheroprotective functions of HSP27 are dependent on  
estrogen and/or its relationship with ER $\beta$



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## **2.0 MANUSCRIPT #1:**

### **NM23-H2, an Estrogen Receptor $\beta$ Associated Protein, Shows Diminished Expression with Progression of Atherosclerosis**

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#### **2.1 Significance of this Manuscript**

This work uncovers a novel role for a known metastasis-suppressor protein, NM23-H2, as an ER $\beta$  co-regulatory molecule that is expressed in the vascular system and may regulate the response to estrogen in these tissues. We show that both estrogen and an ER $\beta$ -specific agonist DPN can induce the expression of NM23-H2 and can cause its translocation to the nucleus. However, although NM23-H2 is expressed in vascular cells in vitro, its abundance in the vessel wall of human coronary arteries diminishes as normal healthy arteries become burdened with atherosclerotic plaque. This may have important consequences in the vascular response to estrogen that is lost with the loss of NM23-H2.

#### **2.2 Author Contributions**

As first author of this manuscript, I was responsible for much of the experimental design and for carrying out most experiments. As I was a junior trainee, Yong-Xiang Chen, the second

author on this manuscript, also carried out some experiments as he trained me in the use of these techniques (e.g. immunolabeling in Figures 2A and 2B). I wrote the majority of the manuscript in conjunction with my supervisor, and was responsible for the reviews to both the written manuscript and the experimental additions throughout the publication process.

## 2.3 Abstract

While estrogen receptor (ER) profile plays an important role in the response to estrogens, receptor co-regulators act as critical determinants of signaling. Although the clinical effects of ovarian hormones on various normal and pathological processes are an active area of research, the exact signaling effects on, for example the vessel wall, are incompletely understood. Hence, we sought to discover proteins that associate with ER $\beta$ , the isoform that shows up-regulated mRNA expression after arterial injury. Using a yeast two hybrid screen we identified NM23-H2, a multi-faceted metastasis suppressor candidate protein, as an ER $\beta$  associated protein. Although NM23-H2 was immunodetected in arteries from young subjects ( $27\pm 6$  years, 14 males and 6 females) with benign intimal hyperplasia, expression was diminished in fatty streaks/ atheromas, and altogether absent in advanced atherosclerotic lesions. Both nm23-H2 mRNA and protein were expressed by vascular cells *in vitro*. Treatment with 17 $\beta$ -estradiol and an ER $\beta$ -selective agonist, DPN, increased protein expression of NM23-H2; an effect that was not seen with an ER $\alpha$  selective agonist, PPT. Estrogen also prompted nuclear localization of NM23-H2 protein in human coronary SMCs. An *in vitro* mimic of inflammation decreased the expression of NM23-H2 in SMCs, which was restored upon addition of estrogen and dependent upon the estrogen receptor. In summary, we report the novel association of NM23-H2 with ER $\beta$ , and show for the first time its expression in vascular cells and demonstrate regulation of its expression and localization by estrogen. In that the abundance of NM23-H2 diminishes with both the advancement of atherosclerosis and inflammation, this ER $\beta$  associated protein may play an important role in mediating the vasculoprotective effects of estrogens.

## 2.4 Introduction

Elevated cardiovascular morbidity and mortality rates among postmenopausal women remain a major health concern in western societies. The most obvious explanation for the relative protection against cardiovascular events in pre-menopausal women is the presence of ovarian hormones. However, large randomized clinical trials fail to demonstrate the expected cardiovascular benefits of hormone replacement therapy (HRT) in post-menopausal women (9,31,32). While there are several important caveats to these clinical studies there is a critical need to better understand how estrogens act at the level of the vessel wall (30).

Estrogens act via two estrogen receptors ( $ER\alpha$  and  $ER\beta$ ), that can exist as either homo- or hetero-dimers (4,10,21). While the two receptors share structural similarities in certain regions (the A/B domain has only 30% sequence identity between  $ER\beta$  and  $ER\alpha$ ), unique proteins that associate with ERs are important in determining the biological effects of the hormone ligand (6,7,10,16,21,28). When activated, the receptors translocate to the nucleus and modulate transcriptional activity through interactions with estrogen response elements (EREs). In addition, these receptors participate in signaling cascades at the cell membrane and therefore have the potential to function entirely independent of gene regulation (13). ERs, as well as ER-associated proteins, may show differential tissue expression patterns and are instrumental in estrogen signaling. In male arteries,  $ER\beta$  is the predominant receptor expressed in the intima, media and adventitia, and data are emerging to suggest its expression is correlated with the degree of atherosclerosis (12). Moreover, the expression of  $ER\beta$  mRNA is markedly up-regulated after vascular injury in male arteries (11,15). Recent evidence demonstrates that  $ER\beta$  expression correlates with the degree of calcification of



atherosclerotic coronary arteries – an effect that is not seen with ER $\alpha$  (2). Therefore, ER $\beta$  plays an important yet unidentified role in atherogenesis and the progression of atherosclerosis. To date, however, there is little information regarding ER $\beta$  associated proteins. For example, Mendelsohn's group discovered Mad2 to specifically interact with ER $\beta$ , however, the functional significance of this interaction remains to be seen (23).

We hypothesized that associated co-regulators of ER $\beta$  may mediate important estrogenic effects in human vascular tissue. To identify candidate proteins we performed a yeast-two hybrid screen using the aforementioned unique A/B region of ER $\beta$ . Recently, we published the first of these findings, demonstrating an interaction between ER $\beta$  and heat shock protein 27 (HSP27), and suggest that this interaction might attenuate ER $\beta$  transcriptional activity (19). Herein, we report the discovery of NM23-H2 as another ER $\beta$  associated protein that is expressed in the artery wall. NM23-H2 is a multi-faceted metastasis suppressor candidate protein that acts as a nucleoside diphosphate kinase, transcriptional regulator and DNA nuclease (24-26). As will be described, there is diminished expression of NM23-H2 with progression of atherosclerotic disease stage. Moreover, we note that NM23-H2 is capable of facilitating estrogen signaling *in vitro*. Hence, it is intriguing to consider that NM23-H2 plays an important role in maintaining vascular homeostasis by mediating the vasculoprotective effects of estrogens.

## **2.5 Materials and Methods**

Briefly, our studies involve two major components: i) the association of NM23-H2 with ER $\beta$  and the regulation of NM23-H2 expression by estrogen; ii) the demonstration of attenuated NM23-H2 expression in atherosclerotic human coronary arteries and SMCs subjected to an inflammatory stimulus.

### **Cell Culture and Treatment**

Human aortic and coronary artery smooth muscle cells (SMCs) and endothelial cells (ECs) of female origin were obtained from Cambrex Bio Science (Walkersville, MD). HeLa and MCF-7 cells were obtained from ATCC (Manassas, VA). Cells were maintained in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, Wisent, Saint-Jean-Baptiste de Rouville, QC), gentamycin (Sabex, Boucherville, QC), and fungizone (Invitrogen). SMCs between passages 5 to 9 were used for all experiments. Prior to all treatments, SMCs were rendered quiescent by serum-starvation (DMEM with 0.5% FBS) for 24 hours. The duration of all 17 $\beta$ -estradiol (E2, Sigma, St. Louis, MO) treatments was 24 hours unless otherwise indicated.

### **RNA Extraction and Northern blotting**

Total RNA was extracted from smooth muscle, endothelial, HeLa, and MCF-7 cells using TRI Reagent (Sigma) as per the manufacturer's instructions. The collected RNA was run on an agarose gel and blotted overnight to a nylon membrane. A randomly primed <sup>32</sup>P labeled probe (New England Biolabs) corresponding to the 650 bp nm23-H2 fragment was incubated

with the nylon membrane overnight at 42°C, and washed five times with low stringency buffer ( $2 \times$  SSC, 0.1% SDS) and twice with high stringency buffer ( $0.1 \times$  SSC, 0.1% SDS). Probed membranes were exposed to film overnight at -80°C.

### **Co-immunoprecipitation**

For the co-immunoprecipitation of endogenous protein, whole cell lysates were collected by harvesting HeLa and SMCs in culture using an immunoprecipitation (IP) buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2mM sodium vanadate, 0.2 mM PMSF, 0.5% NP-40 in PBS) and centrifuging at  $15,000 \times g$  for 20 minutes. Two hundred micrograms of cellular protein, 5 µg of either monoclonal anti-NM23-H2 antibody (Seikagaku, Tokyo) or no antibody (negative control) and 500 µl of IP buffer were incubated at 4°C for 1 hour. Protein-G-agarose beads were then added and agitated at 4°C for 30 minutes. The immunoprecipitated material was washed (3 times in 1X IP buffer) and collected as described above. The final pellet was boiled in SDS loading buffer to remove the agarose beads from the precipitated proteins, and the supernatant was run on an SDS-PAGE gel before the proteins were transferred to a PVDF membrane at 4°C overnight at 20 V. Membranes were then subjected to western blotting using a rabbit polyclonal anti-ERβ (Affinity Bioreagents, Golden, CO; at 1 µg/ml).

### **Protein Harvesting and Western blotting**

Smooth muscle, endothelial, HeLa, and MCF-7 cell lysates were obtained using a RIPA buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, Complete™ inhibitor in PBS]. Cells were washed twice with PBS, lysed in RIPA buffer, collected using a cell scraper, and

placed on ice for 45 minutes. Cell debris was collected by centrifugation at  $15,000 \times g$  for 20 minutes, and the supernatant containing the cellular protein was isolated. Protein was quantified using Bradford reagent (Sigma). For western blotting, 30  $\mu g$  of whole cell protein was loaded onto a 12.5% SDS-PAGE gel and separated at 90 V using gel electrophoresis. Protein was then transferred to a PVDF membrane (BioRad, Hercules, CA) overnight at 20 V, at 4°C. Membranes were then subjected to western blotting using the following antibodies: a rabbit polyclonal anti-NM23-H2 (E.H. Postel; dilution of 1:1000), a rabbit polyclonal anti-ER $\beta$  (Affinity Bioreagents, Golden, CO; at 1 $\mu g/ml$ ), a monoclonal anti- $\alpha$ -actin (Sigma; at 1:250), and a polyclonal anti-DsRed (BD Biosciences).

### **Immunofluorescence**

SMCs in culture were fixed using freshly prepared 4% paraformaldehyde in PBS for 15 minutes on ice. Cell membranes were permeabilized using 0.1% Triton X-100 for 15 minutes, and non-specific proteins were blocked using 2% BSA in PBS for 1 hour. Primary antibodies (anti-NM23-H2, E. Postel; anti-ER $\beta$ , Novus Biologicals, Littleton, CO) were incubated overnight at 4°C (1:200 titre for NM23-H2, 1:100 titre for ER $\beta$ ), washed with PBS, incubated with a secondary antibody (Texas Red tag for ER $\beta$ , fluorescein tag for NM23-H2; Vector Laboratories, Burlington, ON) at a 1:100 dilution for 30 minutes at room temperature (RT), and subsequently washed with PBS. Cell nuclei were counter-stained with Hoechst 33258 (1  $\mu g/ml$ ) for 15 minutes at RT, and cells were visualized on an Olympus BX60 fluorescence microscope.

### **Immunohistochemistry**

Cross sections of normal coronary arteries from 20 individuals who died as a result of non-cardiovascular causes were obtained from the coroner's service at the Vancouver Hospital and Health Sciences Center (Vancouver, Canada) (20). Proximal segments of the left anterior descending (LAD), the left circumflex (LCX), and the right (RCA) coronary arteries were harvested from 14 men (with a total of 34 artery segments) and 6 women (with a total of 17 artery segments) ( $27 \pm 6.4$  years). All tissue specimens were immersion fixed with 10% neutral buffered formalin and embedded in paraffin. These post-mortem arteries were harvested within 6 hours of death, and previously we demonstrated the preserved protein and mRNA content of these arteries (3,8). In order to identify histopathological features of these arteries, sections were stained with hematoxylin and eosin (H&E), Masson's trichrome and Movat's pentachrome stain. Histopathological classification of lesions was done according to the methods of Stary *et al.* (29). Immunolabeling was performed using the following antibodies: a rabbit polyclonal anti-human NM23-H2 (E.H. Postel; dilution 1:200), an EC marker (von Willebrand Factor 8; anti-vWF8, 1:1000, DAKO, Carpinteria, CA), an SMC specific marker ( $\alpha$  smooth muscle actin; anti- $\alpha$ SMA, 1:100, DAKO), an macrophage-specific marker (anti-CD68, 1:100, DAKO), and a T-lymphocyte-specific marker (anti-CD45R, 1:100, DAKO). Biotinylated anti-rabbit or anti-mouse IgG (Vector Laboratories) was used as a secondary antibody. Immunohistochemistry was performed as described previously (1). Briefly, tissue sections were deparaffinized and incubated with 10% horse or goat serum (Vector Laboratories) for 20 minutes to minimize the nonspecific binding of the primary antibody before incubation overnight with one of the primary antibodies in a 4°C moisture chamber. Tissue sections were then incubated with the appropriate secondary antibody for 30 minutes at RT. To inhibit endogenous peroxidase activity tissue sections

were incubated with 3% H<sub>2</sub>O<sub>2</sub> for 30 minutes before incubation with peroxidase-labeled streptavidin (Vector Laboratories) for 30 minutes. Visualization of positive immunolabeling was made possible by the addition of the standard peroxidase enzyme substrate 3,3'-diamino-benzidine tetrahydrochloride (DAB, Sigma), which resulted in a brown colour reaction product. Hematoxylin was used as the nuclear counterstain. PBS washes were used between each of the aforementioned steps. The number of NM23-H2-positive cells in the vascular wall was counted for each section, and then a numerical grade was assigned as follows, according to the number of NM23-H2-positive and ER $\beta$ -positive cells: score of 0(-), no positively-labeled cells; score of 1(+), 1 to 30; score of 2(+), 31 to 60; and score of 3(+),  $\geq 61$  in each section of coronary artery.

For the immunohistochemical positive controls, the tissue blocks retrieved from human breast carcinoma tissue were used for NM23-H2 and ER $\beta$  immunostaining. For negative control, nonimmune mouse and rabbit IgGs were used instead of the respective primary antibodies.

### **Enzyme-Linked Immunosorbent Assay (ELISA)**

The human coronary artery SMCs (HCASMCs) of female origin were grown to confluence in 24-well dishes containing 10% FBS DMEM. The NM23-H2 expression was quantified using an ELISA as previously described (1). Briefly, the cells were treated by lipopolysaccharide (LPS; 5, 10  $\mu$ g/ml; Sigma) with or without E2 (100 nM), E2 (20 nM, 100 nM) with or without ICI 182, 780 (10  $\mu$ g/ml, TOCRIS, Ellisville, MO), a potent ER $\beta$ -selective agonist diarylpropionitrile (DPN, 10nM, TOCRIS) with or without ICI (10  $\mu$ g/ml), and an ER $\alpha$ -selective agonist propylpyrazole-triol (PPT, 10 nM, TOCRIS) with or without

ICI (10 µg/ml) for 24 hours, respectively, and then fixed with 4% paraformaldehyde.

Following triplicate washes in PBS and blocking with 3% skim milk powder for 1 hour the cells were incubated overnight at 4°C with an rabbit anti-NM23-H2 (diluted 1: 1000 in 0.1% BSA/PBS) primary antibodies, washed thrice with PBS and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000 in 0.1% BSA/PBS, Dako) at room temperature (RT) for 30 minutes, respectively. Three washes with PBS were again performed before binding of the second antibody was detected by adding the substrate 3,3',5,5'-tetramethylbenzidine (Sigma). The reaction was stopped by adding 25 µl of sulfuric acid and transferring the media into 96-well plates in order to read the optical density (OD) at 450 nm using a microplate reader (BioRad Laboratories, Hercules, CA). After the media was transferred, the cells were washed with PBS and the cell nuclei were stained with Haematoxylin (Sigma) for 20 minutes at RT. Two areas of each well were photographed using bright microscopy at x100 magnification, and the cell density was determined (i.e., cell number per mm<sup>2</sup>) in order to ensure that differences in OD readings were not simply due to differences in cell number that might have occurred as a result of treating HCASMCs with either LPS with or without E2, either E2 with or without ICI 182, 780, either DPN and PPT. The OD reading measurement was therefore divided by the number of HCASMCs per mm<sup>2</sup> to account for this possibility.

## **Statistics**

Values are means ± SEM. Comparisons of groups was performed using a One-Way ANOVA. Statistical significance was defined by p value <0.05 and is denoted by an asterisk (\*).

## 2.6 Results

### NM23-H2 associates with ER $\beta$

Using a yeast two hybrid screen we identified previously unreported protein interactions with the unique A/B domain of ER $\beta$  (19). From the initial library of  $5.3 \times 10^6$  clones only three clones were found to represent true positive interacting proteins. One of the clones was identified as nm23-H2, as it shared 98.7% sequence identity with the reported NM23-H2 protein sequence in the open reading frame (Swiss-Prot Accession No. P22392).

### Vascular Expression and Interaction of NM23-H2 with ER $\beta$

As we are interested in the potential of NM23-H2 to modulate vascular effects of estrogen, we began by determining the expression profile of this protein in vascular cells in culture. NM23-H2 expression was examined in endothelial cells (ECs) and smooth muscle cells (SMCs). Both HeLa and breast tumor-derived MCF-7 cells are known to express high levels of NM23-H2 and thus served as positive controls (14,22). Northern blot analysis demonstrated the presence of a 650 bp mRNA in all of the cell types investigated (**Figure 2.1A**). Similarly, protein was isolated from the aforementioned cell types (as well as quiescent SMCs) and subjected to western blot analysis with an NM23-H2 specific antibody. All cell types contained the 17.5 kDa endogenous protein (**Figure 2.1B**). A higher molecular weight species of approximately 21 kDa was also observed in MCF-7 and HeLa cells - most likely representing NM23-H1 that may be detected with this polyclonal antibody (unpublished data: EHP).



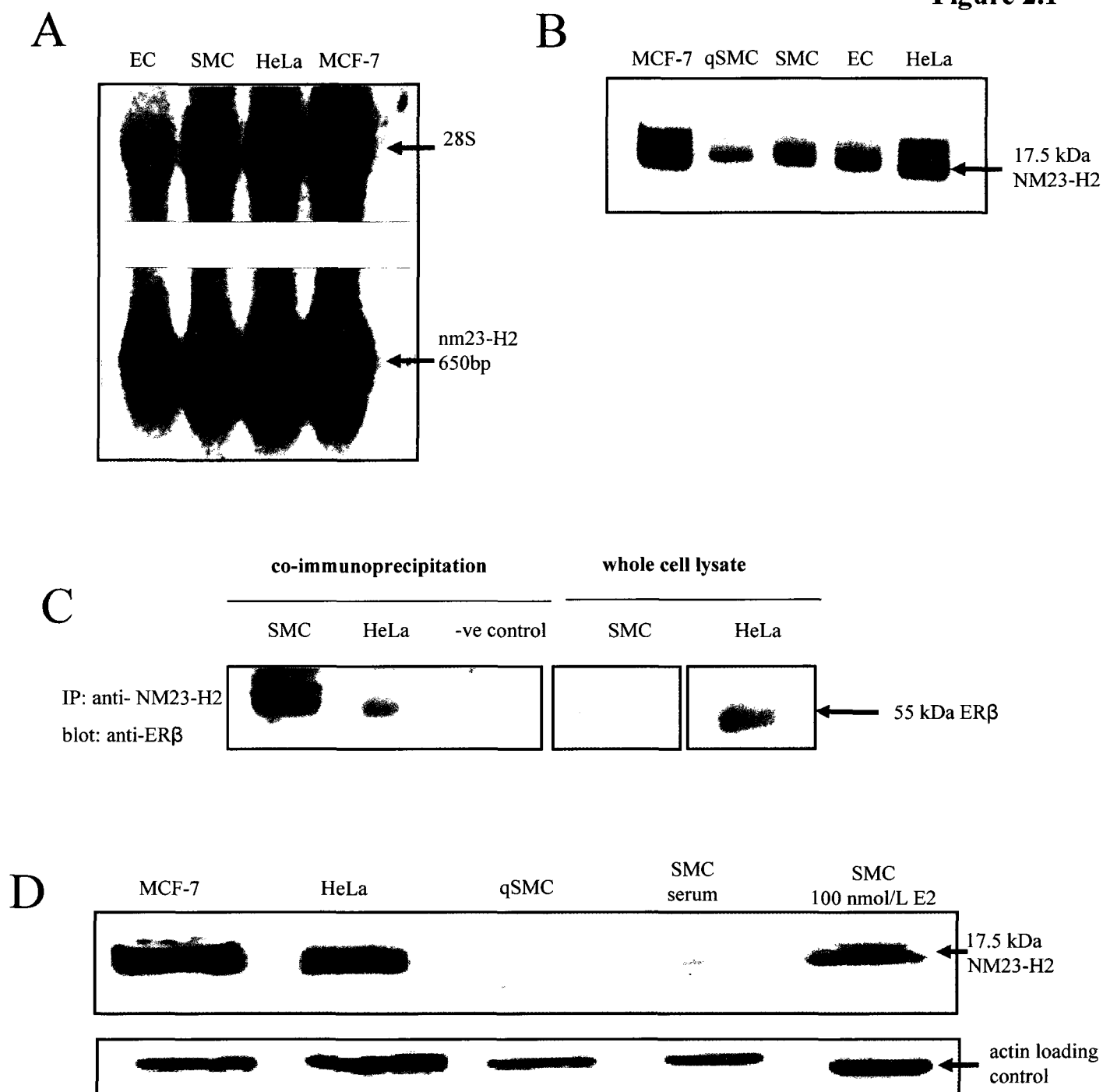
**Figure 2.1: Vascular Expression of NM23-H2**

ECs, SMCs (both quiescent and serum-stimulated), HeLa and MCF-7 cells were examined for NM23-H2 expression.

**A)** Northern blot showing expression of the 650 bp mRNA nm23-H2 transcript and 28S ribosomal RNA.

**B)** Western blot using antibodies specific for the 17.5 kDa NM23-H2 protein.

**Figure 2.1**



To test whether ER $\beta$  and NM23-H2 interact endogenously (i.e. in unmodified primary cells), NM23-H2 was immunoprecipitated from non-transfected SMCs and HeLa cells before being analyzed by western blotting using an ER $\beta$  antibody. The NM23-H2 specific antibody immunoprecipitated the 55 kDa ER $\beta$ , with a band that was more prominent in SMCs compared to HeLa cells (**Figure 2.1C**). A higher molecular weight band was also noted, and corresponded to a non-specific interaction with the beads (e.g., also seen in negative control lane). Analysis of the residual flow-through lysate showed little ER $\beta$  remaining unbound following co-immunoprecipitation (data not shown). Analysis of whole cell lysates from both SMC and HeLa cells consistently demonstrated expression of ER $\beta$  - thereby acting as a positive control for these experiments.

To determine the ability of estrogen to modulate the expression of NM23-H2, SMCs from quiescent, serum-stimulated and estrogen-stimulated cells were harvested and subjected to western blot using an antibody to NM23-H2. Estrogen treatment resulted in an increase in protein expression when compared to both quiescent and serum-stimulated cells (**Figure 2.1D**). These results indicate the ability of estrogen to regulate the expression of NM23-H2 in SMCs, prompting us to further examine its role in ER $\beta$  and estrogen signaling in vascular cells. To examine the functional consequences of the ER $\beta$ /NM23-H2 interaction, we performed co-localization studies using fluorochrome labeled ER $\beta$  and NM23-H2 antibodies in serum-stimulated and estradiol-treated SMCs. Both ER $\beta$  and NM23-H2 were expressed and co-localized in the nucleus and cytoplasm of SMCs (**Figure 2.2A**).

**Figure 2.1:**

**C)** Co-immunoprecipitation of ER $\beta$  using an NM23-H2-specific antibody for the pulldown and an ER $\beta$ -specific antibody for the detection. Omission of NM23-H2 antibody constituted the negative control (-ve control). Whole cell lysate from both HeLa and SMCs were probed using antibodies to ER $\beta$ .

**D)** Western blot showing HeLa and MCF-7 cells, quiescent (qSMC) and serum-stimulated SMCs (10% serum) and SMCs treated with serum supplemented with 100 nmol/L 17 $\beta$ -estradiol (100 nmol/L E2). Antibodies specific for the 17.5 kDa NM23-H2 protein (top panel) and  $\alpha$ -actin (bottom panel, loading control) were used for immunodetection. SMC expression of NM23-H2 is upregulated in response to E2.

## NM23-H2 Expression in Human Coronary Arteries

To gain insight into the potential *in vivo* role of NM23-H2 in vascular cells, we determined the pattern of expression of NM23-H2 in both normal and diseased human coronary arteries using specific anti-NM23-H2 antibodies. Arteries with complex atherosclerotic lesions were devoid of NM23-H2 expression (data not shown), therefore we studied coronary arteries from subjects less than age 40 years with either no histological evidence of atherosclerosis or minimal lesions that are the precursors of advanced disease. Of the 51 coronary artery cross sections studied, 18 had diffuse intimal thickening (a normal developmental finding that does not obstruct blood flow), 26 had non-obstructive fatty streaks (type I and type II lesions), and 7 sections were considered to have type III or type IV atherosclerotic lesions. NM23-H2 expression was detected in both the endothelium and SMC layers of arteries with diffuse intimal thickening, but only the endothelium was immunopositive for NM23-H2 in those arteries with fatty streaks and/or more advanced atheromas (**Figure 2.2B**). Intimal and medial expression of NM23-H2 was assessed in a semi-quantitative manner and found to diminish with the progression of atherosclerotic disease stage (**Figure 2.2C**). The immunolabeling pattern for NM23-H2 was not individual specific, but rather dependent on the histopathology of the coronary artery. Interestingly, the attenuation of NM23-H2 expression in the intima (but not media) of these coronary arteries coincided with a decrease in ER $\beta$  expression (**Figure 2.2C**). Given our interest in atherogenesis and the potential disparity between the sexes, we analyzed the differences in NM23-H2 expression in male versus female proximal left anterior descending coronary arteries from young patients. As demonstrated in **Figure 2.2D**, there is a trend towards an increase in the number of NM23-H2 positive sections in females versus males, in all three layers of the vessel wall

**Figure 2.2: NM23-H2 and Estrogen Signaling and Expression in Human Coronary Arteries**

**A)** Human SMCs in culture were plated, and 24 hours later were stimulated with 10% serum (10% serum SMC, left column) or 10% serum plus 100 nmol/L 17 $\beta$ -estradiol (100 nmol/L E2 SMC, right column) for 30 minutes. Cells were immunolabeled with fluorescent antibodies to ER $\beta$  (red), NM23-H2 (green), and a Hoescht stain for the nuclei (blue). Merged photos show both green and red output signals. Original magnifications were x400.

**B)** Immunohistochemical analysis of human coronary arteries: Movat pentachrome stain (top row), smooth muscle  $\alpha$ -actin (SMA, second row), von Willebrand Factor 8 (vWF8, third row) and NM23-H2 (fourth row). Tissue sections are from arteries without atherosclerosis but showing benign diffuse intimal thickening (DIT, left column), and atherosclerotic arteries with fatty streaks (type-I lesion, middle column) and atheromas (type-III lesion, right column). NM23-H2 immunopositive endothelium is denoted by an arrow head; NM23-H2 immunopositive smooth muscle cells are denoted by an arrow. Original magnification: lower power (x20), high power (x400). M = media, I = intima, and L = lumen.

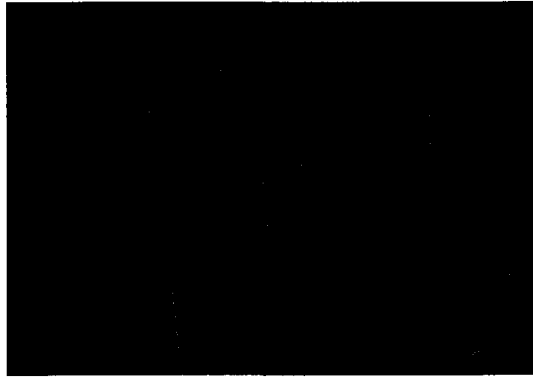
**C)** Semi-quantitative analysis of the % NM23-H2 immunopositive (top row) and % ER $\beta$  immunopositive (bottom row) sections in the medial (left bar graphs) and intimal layers (right bar graphs) of tissue sections described above (\*\*  $p \leq 0.001$  and \*  $p \leq 0.05$  compared to DIT).

**D)** Semi-quantitative analysis of the NM23-H2 expression (left) and ER $\beta$  expression (right) in left anterior descending coronary artery sections, in males versus females. The number of sections determined to be NM23-H2 positive (NM23-H2 +) or ER $\beta$  positive (ER $\beta$ +) in all sections were counted and expressed relative to the total number of sections. (males = black bars; females = open bars).

Figure 2.2

A

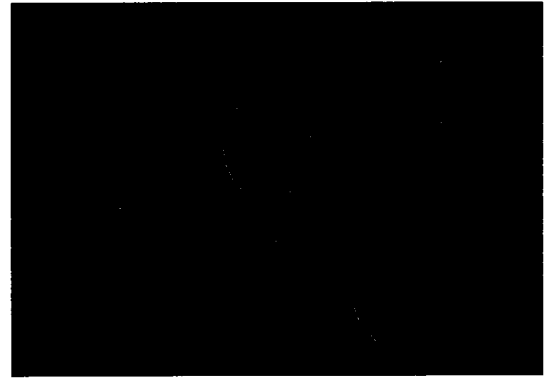
NM23-H2



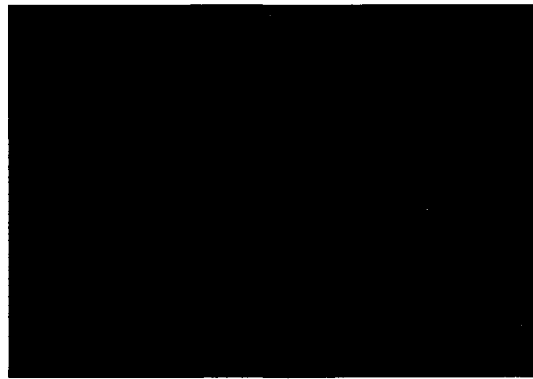
ER $\beta$



nuclei



merged



10% serum SMC

100 nmol/L E2 SMC

Figure 2.2

B

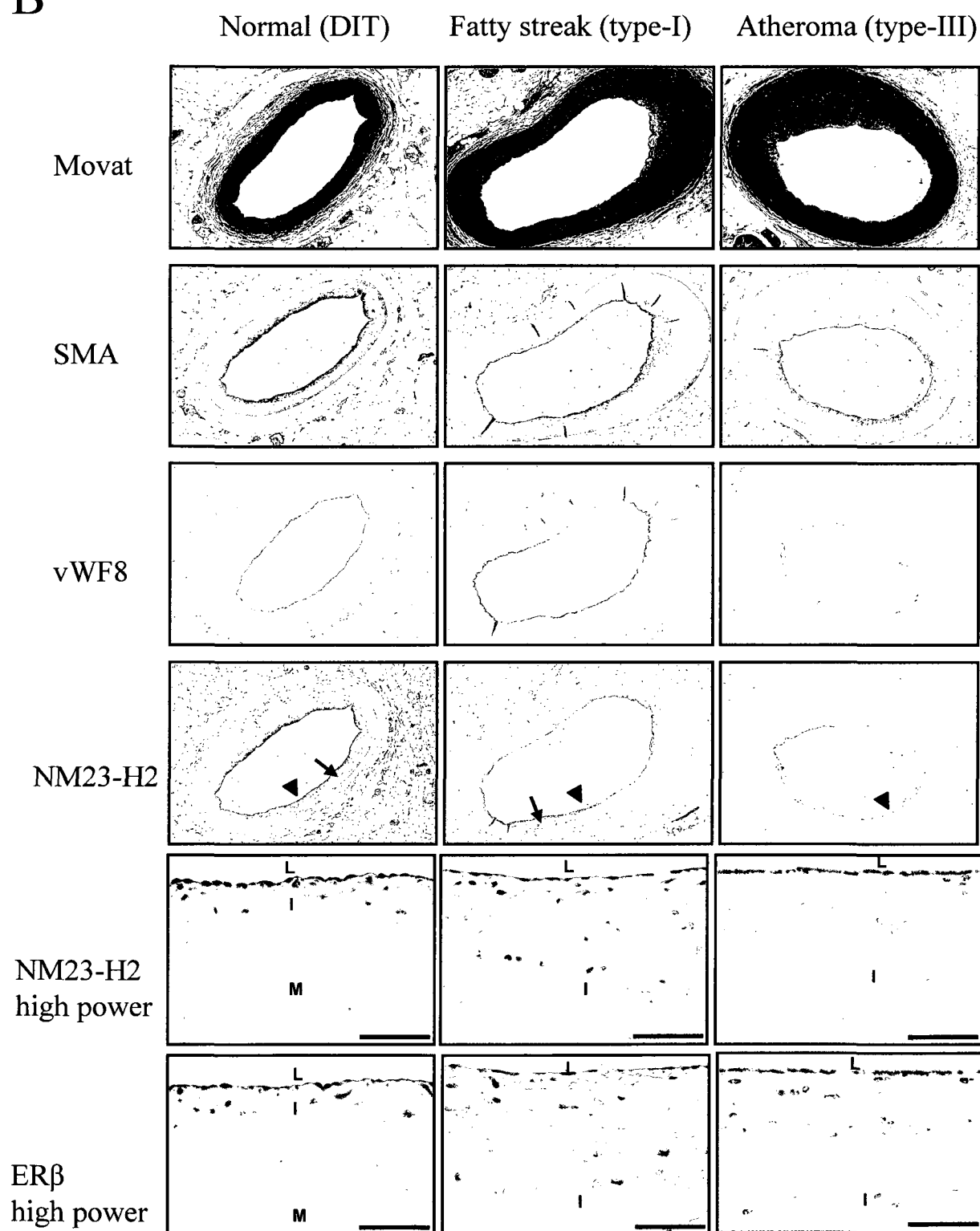
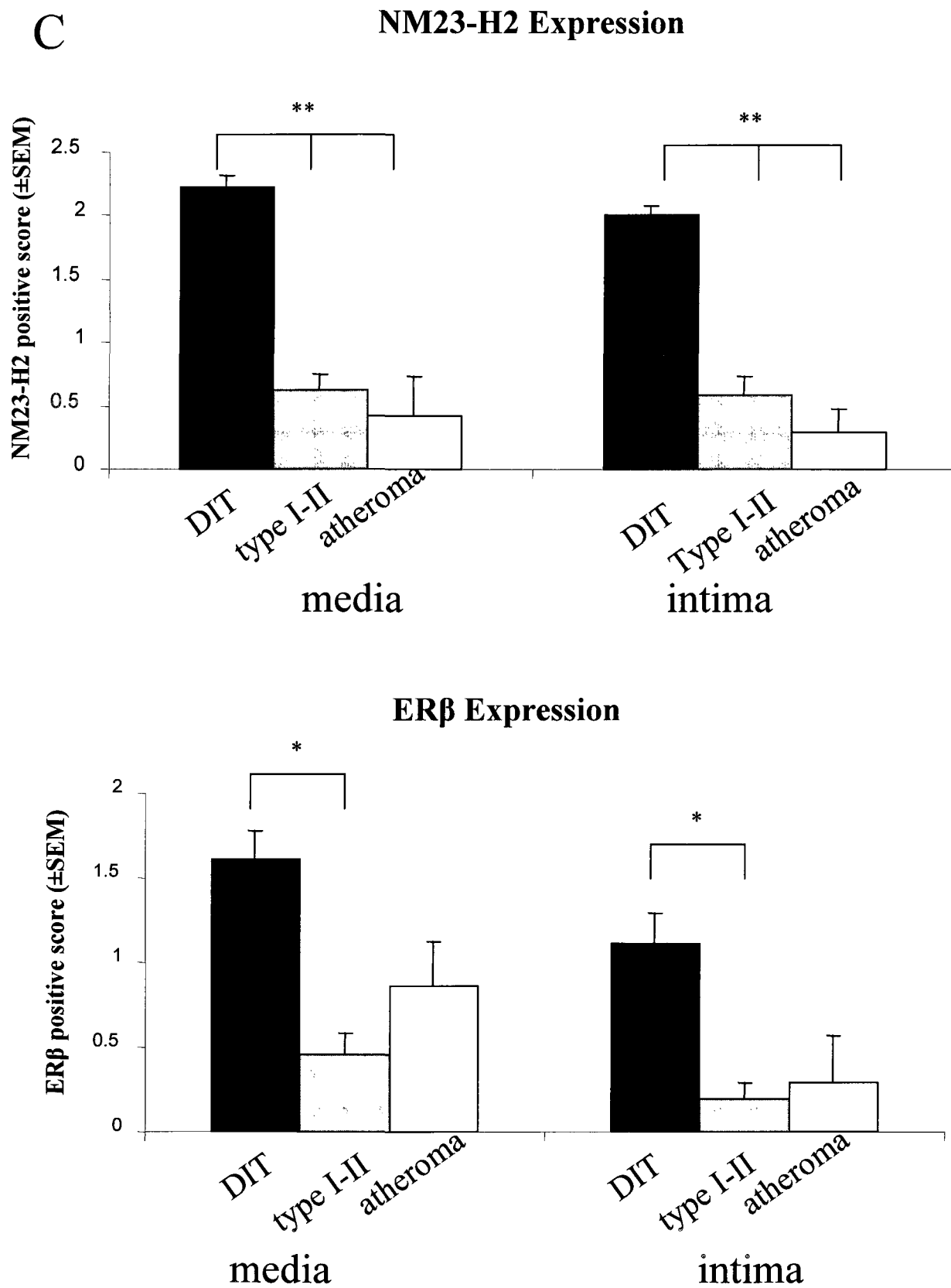


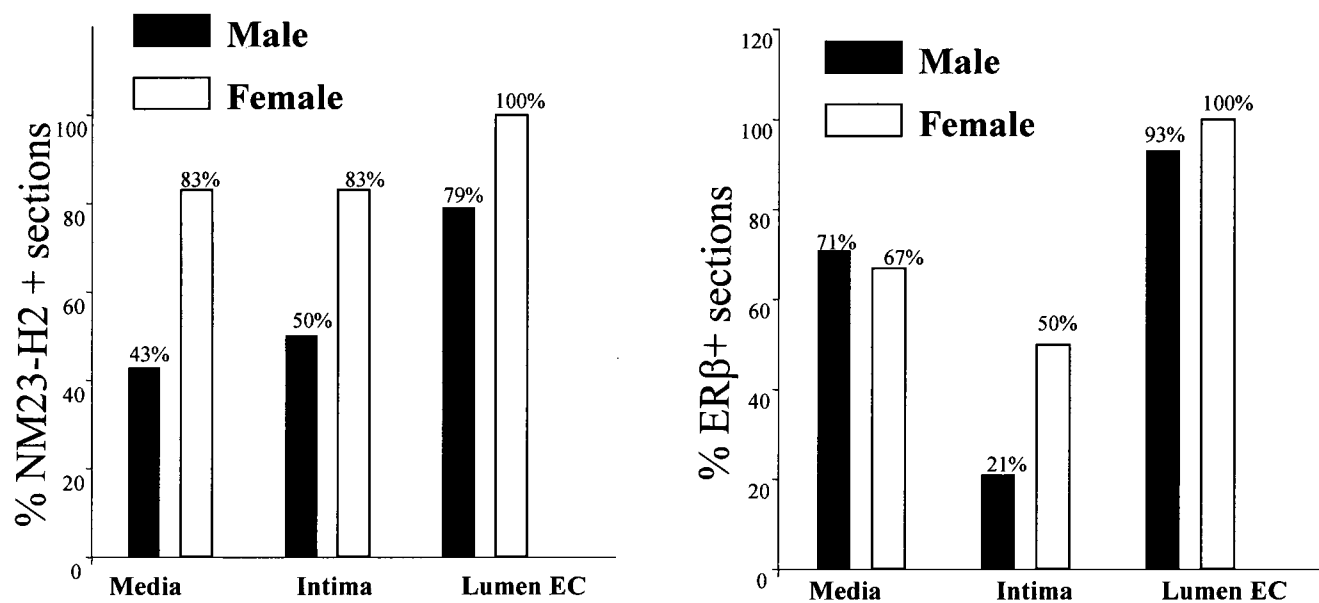


Figure 2.2



**Figure 2.2**

**D**



**Table 2.1**

Analysis of lesion types for the cross sections used for NM23-H2 and ER $\beta$  analysis. Normal DIT sections (left column) or atherogenic sections (type I-V, right column) were counted and expressed relative to the total number of sections. M = male, F = female.

**Table 2.1****Lesion Type**

| <b>Gender</b> | <b>DIT</b> | <b>I-II</b> | <b>III-V</b> | <b>TOTAL<br/>(n=)</b> |
|---------------|------------|-------------|--------------|-----------------------|
| <b>M</b>      | 14% (2/14) | 50% (7/14)  | 36% (5/14)   | <b>14</b>             |
| <b>F</b>      | 50% (3/6)  | 33% (2/6)   | 17% (1/6)    | <b>6</b>              |

(media 83% vs. 43%; intima 83% vs. 50%, and luminal ECs 100% vs. 79%). We examined the ER $\beta$  expression in these arteries and compared male versus female subjects (**Table II**). There was no difference between the sexes in ER $\beta$  expression in the media and luminal ECs, but a trend toward an increase in ER $\beta$  expression in the intima (21% in males versus 50% of females), paralleling NM23-H2 expression. However, conclusions from this analysis can not be drawn due to the low sample number of young subjects in this study.

### **Estrogen Effects on NM23-H2 in Human Coronary Artery SMCs**

As atherosclerosis is an inflammatory disorder, we sought to determine the influence of an *in vitro* mimic of inflammation, LPS, on NM23-H2 expression. Increasing doses of LPS reduced SMC NM23-H2 protein expression – an effect that was partially reversed with the addition of E2 (**Figure 2.3A**). Increasing doses of estrogen progressively augmented NM23-H2 expression - an effect that was blocked by the ER antagonist ICI 182,780 (**Figure 2.3A**). Furthermore, the increasing expression of NM23H2 in HCASMCs was only observed by using a selective ER $\beta$  agonist DPN but not an ER $\alpha$ -selective agonist PPT. Finally, this effect of ER $\beta$ -mediated induction of NM23-H2 expression was blocked by the ER antagonist ICI 182,170 (**Figure 2.3A**).

To investigate the potential role of NM23-H2 to modulate estrogen signaling via ER $\beta$  we performed the following *in vitro* experiments. As endothelial cell NM23-H2 expression in human coronary arteries persisted throughout the progression of atherosclerosis, we surmised that SMCs may be the target of estrogen regulation by NM23-H2 in the vessel wall.

Treatment of SMCs with E2 up-regulated NM23-H2 protein expression and showed

### **Figure 2.3: NM23-H2 Expression and Localization in HCASMCs *in vitro***

#### **A) NM23-H2 Expression in Response to Inflammatory Stimuli and Estrogens**

NM23-H2 expression was analyzed using an enzyme-linked immunosorbent assay (ELISA) in cultured human coronary artery smooth muscle cells (HCASMCs). Cells were treated with or without LPS and 17 $\beta$ -estradiol (left panel), 17 $\beta$ -estradiol with or without ICI 182,780 (middle panel), a potent ER $\beta$ -selective agonist DPN and an ER $\alpha$ -selective agonist PPT with or without ICI 182,780 (right panel), respectively. Expression was measured as a function of optical density at 450nm and normalized to cell number/mm<sup>2</sup>. NS: not significant.

#### **B) Immunofluorescence of NM23-H2 in HCASMCs Treated with or without 17 $\beta$ -estradiol**

HCASMCs in culture labeled with anti-NM23-H2 antibodies (green; left panel) and Hoechst 33258 nuclear stain (blue, right panel) after 30 minutes of serum stimulation alone (control; top) or with the addition of 17 $\beta$ -estradiol (100 nmol/L E2; bottom). Shown are NM23-H2 positive and negative nuclei. Original magnifications were x400.

#### **C) 17 $\beta$ -estradiol Promoted NM23-H2 Immuno-Positive Nuclear Localization**

NM23-H2 positive nuclei were counted, and expressed as a percentage of total number of nuclei per high power field (magnification was x200). Cells were counted after 30 minutes or 24 hours of estradiol treatment, and compared to cells treated with serum alone. Multiple fields with multiple cells were used for analysis.

A

NM23-H2  
Expression

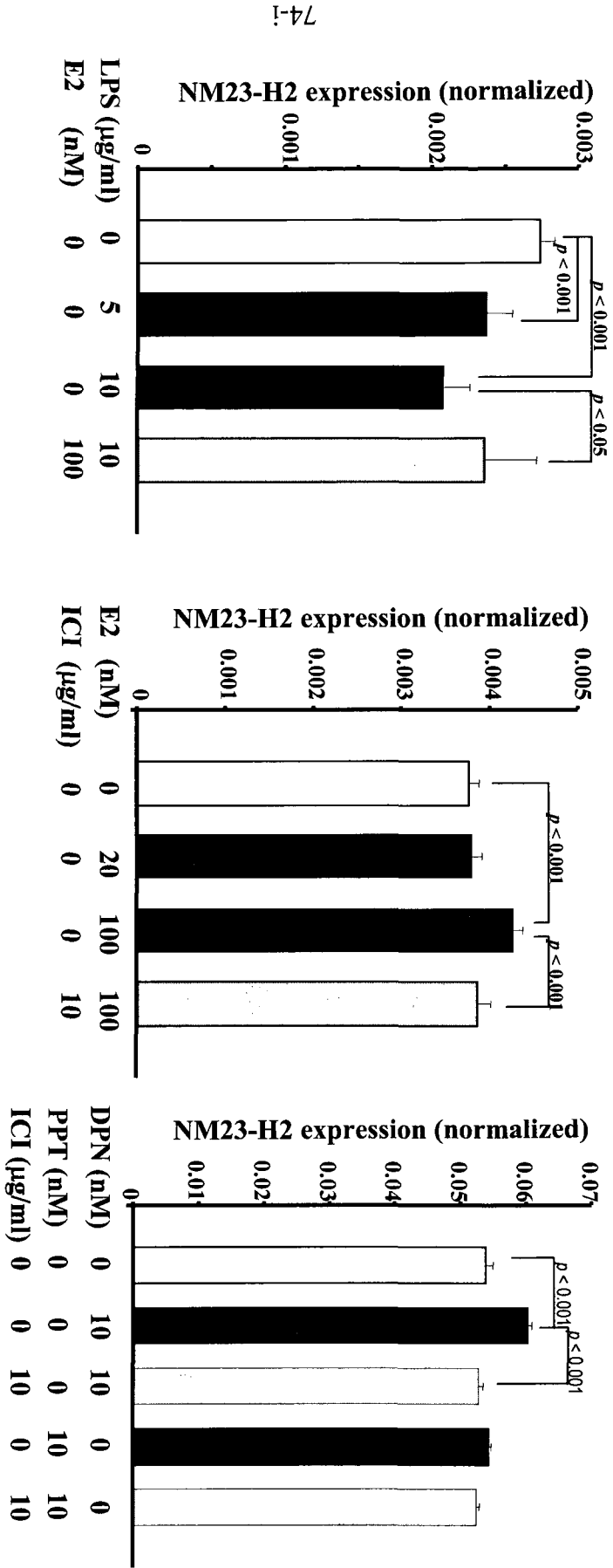
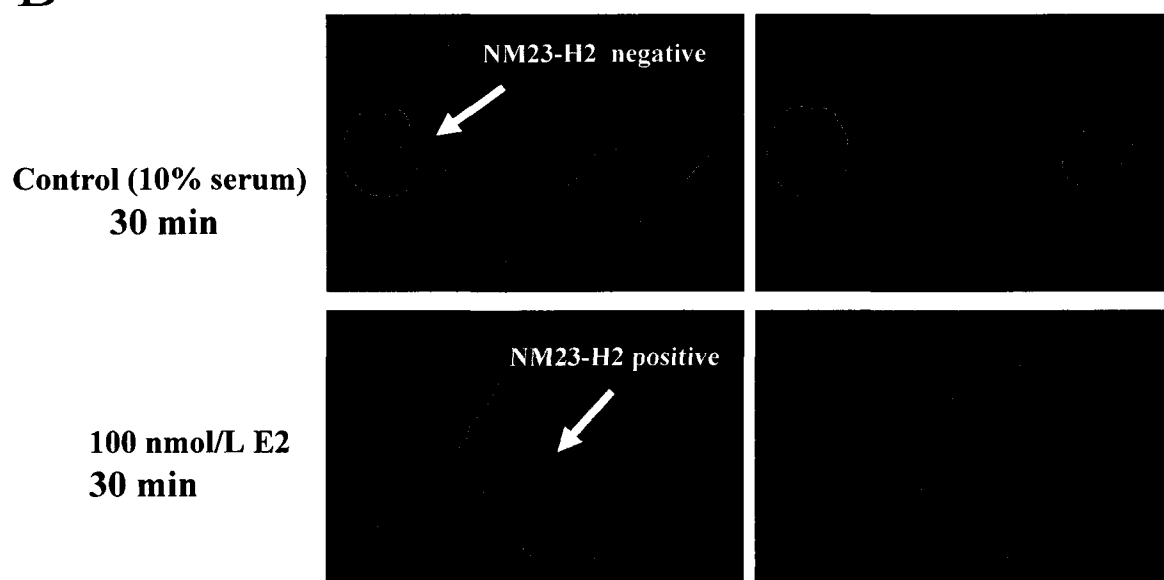


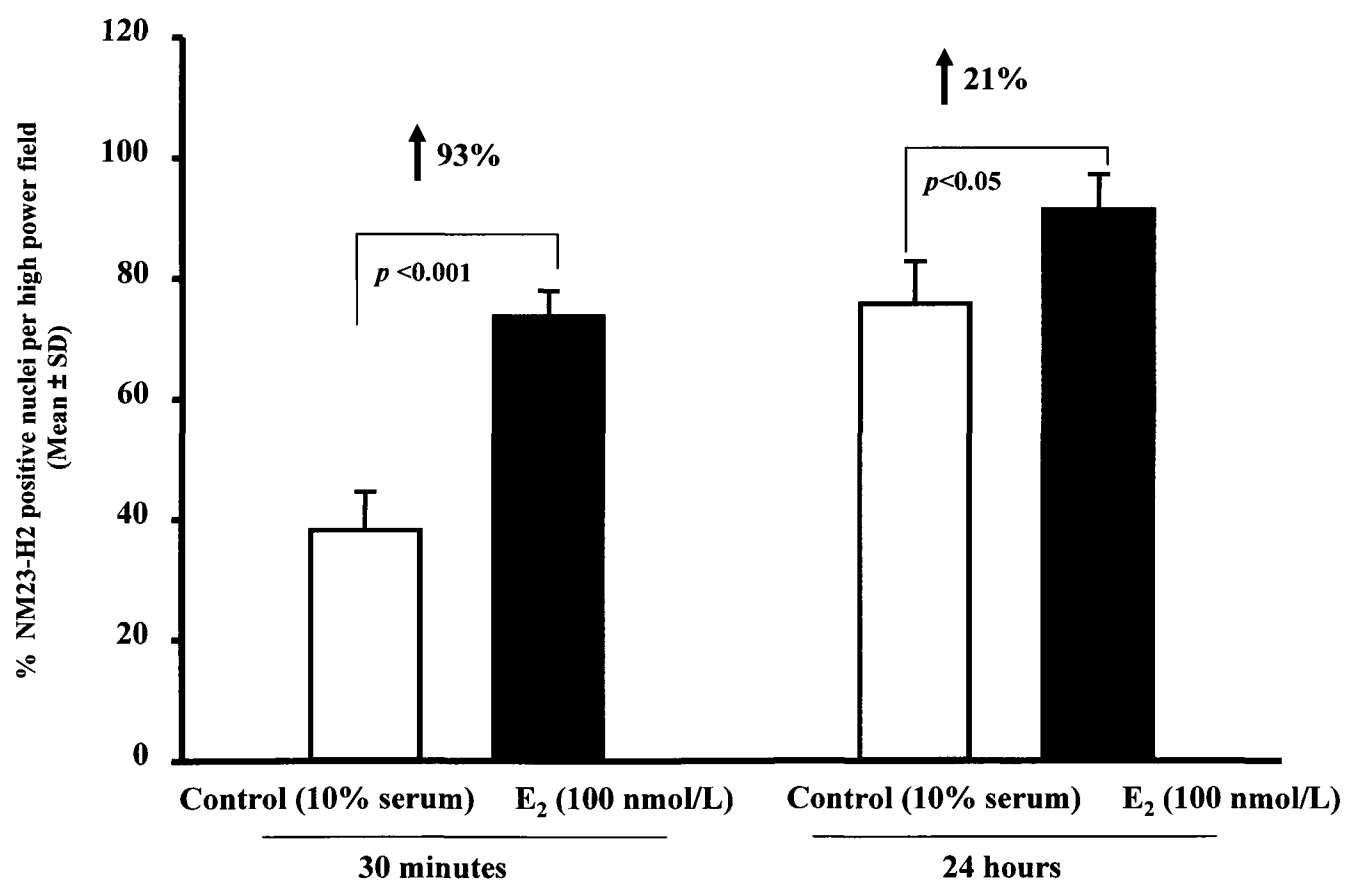
Figure 2.3

Figure 2.3

B



C





increased nuclear localization of NM23-H2 compared to cells treated with serum alone. Therefore, cells were treated with either serum alone or serum supplemented with E2, and the number of NM23-H2 positive nuclei were counted and expressed relative to total number of nuclei per high power field (**Figure 2.3B** and **2.3C**). As anticipated from our initial observations, SMCs treated with E2 for 30 minutes showed 93% more NM23-H2 immunopositive nuclei compared to cells treated with serum alone ( $p < 0.001$ ). This effect was diminished after 24 hours with only 21% more NM23-H2 immunopositive nuclei in E2-treated cells compared to control ( $p < 0.05$ ). Therefore, treatment of SMCs with E2 promptly increased the nuclear translocation of NM23-H2 compared to cells treated with serum alone.

## 2.7 Discussion

Physiologic and pathologic control of the response to steroid hormones is critically linked to the relative abundance of specific regulatory proteins that associate with their respective receptors (5). As we are interested in the potential role of estrogens in vascular disease, we sought to identify and characterize proteins that might associate with ER $\beta$ , the ER isoform that is known to be correlated with atherosclerosis in male arteries and whose mRNA up-regulated in response to arterial injury (11,12,15). By means of a yeast two hybrid screen, we discovered the novel interaction of the unique A/B domain of ER $\beta$  with NM23-H2 and confirmed this interaction using three separate *in vitro* assays.

Endogenous NM23-H2 and ER $\beta$  co-localize and interact in vascular SMCs. Interestingly, in separate immunolabeling studies with human arteries and breast tumors ER $\alpha$  did not co-localize with NM23-H2 or ER $\beta$  (data not shown). While NM23-H2 expression is absent in coronary arteries with advanced atherosclerosis, it was found primarily in intimal SMCs of

post-mortem coronary arteries from young individuals with non-obstructive disease. The distinct attenuation in NM23-H2 expression in intimal SMCs occurred with progression of atherosclerotic disease stage and coincided with a decrease in ER $\beta$  expression. When NM23-H2 expression was analyzed in the context of gender difference, there is a trend towards an increase in NM23-H2 protein expression in female versus male arteries, and while these results are not statistically significant, they prompt further study. For example, if estrogen increases the expression of NM23-H2, there may be a sex specific difference in NM23-H2 expression. In this study, there was a disproportionate number of atherogenic lesions in male samples compared to female samples (86% versus 50%  $p=0.13$ ), in part reflecting the higher incidence of earlier onset of disease progression in the male population. Thus, the higher levels of NM23-H2 expression in female arteries may simply be a reflection of the lower disease burden.

Given the important role of inflammation in atherogenesis, we showed that the inflammatory mediator, LPS, reduced NM23-H2 expression thereby mirroring the attenuated expression in atherosclerotic arteries. Interestingly, 17 $\beta$ -estradiol supplementation partially restored SMC expression of NM23-H2 – an effect which was dependent upon the estrogen receptor. NM23-H2 expression was only increased in response to a highly selective ER $\beta$  agonist, DPN, but not with an ER $\alpha$ -selective agonist. Moreover, 17 $\beta$ -estradiol upregulated SMC expression of NM23-H2 and prompted its nuclear translocation. Hence, we surmised that estrogens may modulate the regulatory role of NM23-H2 in estrogen signaling. Experiments that specifically address this hypothesis are ongoing.

Taken together, these data suggest that NM23-H2 may be an important regulator of vessel wall homeostasis and estrogen responsiveness. While we acknowledge the limitations of

these studies as to a causal relationship between NM23-H2 and atherosclerosis, as an ER $\beta$  co-activator and potentiator of the anti-migratory effects of 17 $\beta$ -estradiol, it follows that early attenuation of NM23-H2 expression with advancement of atherosclerosis would result in a reduction in the salutary effects of estrogens on atherogenesis. Although 17 $\beta$ -estradiol may augment NM23-H2 levels, this effect may be lost with the reduction in estrogen levels that occurs with the onset of menopause. Theoretically HRT should be capable of restoring NM23-H2 expression levels; however there may be a window of opportunity for the initiation of HRT – beyond which the beneficial effects on NM23-H2 expression are less pronounced or lost. Certainly, in clinical practice, HRT is often initiated in women who are already several years post-menopause (17,18). Hence, understanding the role of NM23-H2 and ER $\beta$  as a woman approaches and enters menopause may be very important in appreciating the therapeutic profile of HRT. For example, if the NM23-H2 pathway has ceased to function and cannot be re-activated, HRT may actually produce unwanted side-effects (e.g. hypercoagulable state) (27). Finally, while these data highlight the specific vascular interplay between NM23-H2 and ER $\beta$ , we cannot exclude the possibility that other co-regulatory proteins, as well as alternate ERs forms (e.g. ER $\alpha$ / $\beta$  heterodimers or ER $\alpha$  homodimers) also contribute to vessel wall homeostasis and require further study.

## **2.8 Acknowledgments**

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### **3.0 MANUSCRIPT #2:**

#### **Discovery of NM23-H2 as an Estrogen Receptor $\beta$ Associated Protein: Role in Estrogen-Induced Gene Transcription and Cell Migration**

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#### **3.1 Significance of this Manuscript**

We identify in this study that NM23-H2 is an ER $\beta$  co-regulatory molecule found to interact via yeast two hybrid, and confirmed using an over-expressing system and at endogenous levels in various cell lines. These two proteins co-localize both *in vitro* in HeLa and MCF-7 cells, as well as *in vivo* in breast carcinoma tissue. We also show that NM23-H2 is capable of acting as a co-activator of estrogen-mediated gene transcription via ER $\beta$ , and that NM23-H2 acts synergistically with estrogen to inhibit cell migration. These results identify a novel ER $\beta$ -specific co-regulatory molecule that is found to co-localize with ER $\beta$  and may have important functions in regulating estrogen-mediated gene activation and cell migration.

#### **3.2 Author Contributions**

As first author of this manuscript, I was responsible for a major portion of the experimental design and for carrying out most experiments. Again since I was a junior trainee, Yong-



Xiang Chen, the second author on this manuscript, also carried out some experiments and trained me in the use of these techniques (e.g. immunolabeling in Figures 2A and 2B). The other co-authors either contributed via their training as I first began this project, gave helpful insight into the written portion of the manuscript, or contributed to the initial work with the yeast two hybrid assay (which was performed prior to my arrival in the lab). I wrote the majority of the manuscript in conjunction with my supervisor, and was responsible for the reviews to both the written manuscript and the experimental additions throughout the publication process.

### 3.3 Abstract

The regulation of the estrogenic responses may be influenced by the proteins that associate with estrogen receptors (ERs) rather than solely with the receptors themselves. ER $\beta$  is expressed in blood vessels and may play an important role in vascular disease. We hypothesized that specific proteins interact with ER $\beta$  to modulate its response to estrogens. By means of a yeast two hybrid screen, we discovered that NM23-H2, a multi-faceted protein associates specifically with ER $\beta$ . NM23-H2 and ER $\beta$  consistently co-localize in a variety of human tissues (e.g. breast tissue), whereas ER $\alpha$  and NM23-H2 did not co-localize. Estrogen response element-mediated transcription increased by 97% when NM23-H2 and ER $\beta$  were over-expressed in MCF-7 cells ( $p \leq 0.001$ ). Moreover, there was a synergistic effect of NM23-H2 over-expression with estrogen treatment on the reduction of MCF-7 cell migration ( $p \leq 0.001$ ). These results suggest that NM23-H2 associates with ER $\beta$  and is capable of modulating estrogen-induced gene transcription, as well as cell migration. Hence, NM23-H2 may play an important role in modulating the response to endogenous and exogenous estrogens, perhaps even within the context of vascular disease.

### 3.4 Introduction

The biological effects of estrogen are mediated by at least two cellular receptors: ER alpha ( $ER\alpha$ ) and ER beta ( $ER\beta$ ), that belong to the classical steroid hormone receptor superfamily (15,25,38). When activated, the receptors translocate to the nucleus and modulate transcriptional activity through interactions with estrogen response elements (EREs). These receptors also participate in signaling cascades at the cell membrane, suggestive of a function entirely independent of gene regulation (31). Structurally,  $ER\alpha$  and  $ER\beta$  are subdivided into several functional domains including ligand binding, DNA binding, and both ligand-independent (AF-1) and ligand-dependent (AF-2) activation domains (25, 38). While the two receptors share considerable structural similarities, they derive functional specificity via differential tissue expression patterns and regions of structural diversity (e.g. the A/B domain where there is only 30% sequence identity between  $ER\beta$  and  $ER\alpha$ ) (17,34). Moreover, ER ligand complexes produce different effects in different cells due to variable expression of co-regulatory proteins (e.g. co-activators and co-repressors). Indeed, in some instances the physiological and pathophysiological response to hormones may reside with these co-regulatory molecules, rather than solely with the receptors themselves. Approximately 300 NR associated proteins are known, typically as a result of yeast two hybrid screens that employ the NR as “bait” (*cf.* review by Smith and O’Malley) (52). In general, these proteins do not bind DNA directly, but instead facilitate the interaction of hormone receptors with DNA and other structural proteins – ultimately serving to facilitate (activator) or hinder (repressor) the activation of transcription (16,52).

For a variety of reasons ER $\beta$  has emerged as a key receptor in the vessel wall. For example, the expression of ER $\beta$  mRNA is markedly up-regulated after vascular injury in male arteries (27,33). Moreover, in male arteries, ER $\beta$  is the predominant receptor expressed in the intima, media and adventitia, and its expression correlates with the degree of calcification – a marker of severe atherosclerosis (6,28). Therefore, ER $\beta$  appears to play an important yet unidentified role in the progression of atherosclerosis. In this study we hypothesized that associated co-regulators of ER $\beta$  may mediate estrogenic effects in human vascular tissue. To objectively identify potential associated proteins, we performed a yeast-two hybrid screen using the aforementioned unique A/B region of ER $\beta$ . Heat shock protein-27 (HSP-27) was the first ER $\beta$  associated protein that we studied (35). HSP-27 attenuated ER $\beta$  transcriptional activity, preserved endothelial cell homeostasis, and normal volunteers had >3-fold higher serum levels than those patients with angiographic evidence of coronary artery disease (5). Herein, we report the discovery of NM23-H2 as another ER $\beta$  associated protein. NM23-H2 is a multi-faceted protein that, amongst other roles, acts as a nucleoside diphosphate kinase, transcriptional regulator and DNA nuclease (45-47). Recently, we noted progressive attenuation of NM23-H2 expression in human coronary arteries as the stage of atherosclerosis advanced, as well as in the presence of inflammation (48). We now note *in vitro* that NM23-H2 facilitates estrogen induced gene transcription and acts synergistically with estrogen to attenuate cell migration. Hence, it is intriguing to consider that NM23-H2 may play an important role in vascular homeostasis and perhaps has a critical role in determining responsiveness to estrogens.

### **3.5 Materials and Methods**

#### **Yeast Two Hybrid Assay**

For complete experimental details on the yeast two hybrid screen, please see Miller et al., 2005 (35). Briefly, the ER $\beta$  A/B domain served as a ‘bait’ to identify interacting proteins (or ‘prey’) expressed from a human lung fibroblast cDNA library. Lung tissue was specifically targeted because of its 3-fold higher levels of ER $\beta$  compared to ER $\alpha$  (24). All interactions were conducted in triplicate to ensure the validity of the interaction. Positive clones were sequenced and identified with BLAST, an alignment search tool (<http://www.ncbi.nlm.nih.gov/BLAST/>). All protein sequences were translated and aligned with the Expert Protein Analysis System translational tool and SIM alignment tool; respectively (ExPaSy) (<http://ca.expasy.org/>).

#### ***In vitro* Protein Pulldown Assay**

Yeast two hybrid protein-protein association results were confirmed *in vitro* by a fusion protein pulldown assay. The ER $\beta$  A/B domain was subcloned into a maltose binding protein (MBP) fusion vector (New England Biolabs, Beverly, MA) and expressed in bacteria. The MBP-ER $\beta$  protein was first purified on amylose-agarose beads (20 mg MBP-ER $\beta$  protein per column) and bead-bound fusion protein was then used to test the interaction with an NM23-H2-intein fusion protein (20 mg NM23-H2-intein protein, New England Biolabs). Columns were washed (20 mM Tris pH 8.0, 0.2 M NaCl, 1 mM EDTA, 20  $\mu$ M PMSF) and the detection of retained protein was accomplished by elution with maltose followed by SDS-gel

electrophoresis and immunoblotting with an appropriate antibody (IMPACT system; New England Biolabs).

### **Cell Culture and Treatment**

HeLa and MCF-7 cells were obtained from ATCC (Manassas, VA). Cells were maintained in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, Wisent, Saint-Jean-Baptiste de Rouville, QC), gentamycin (Sabex, Boucherville, QC), and fungizone (Invitrogen). Cells were treated with 17 $\beta$ -estradiol (Sigma, St. Louis, MO) for 24 hours unless otherwise indicated.

### **Co-immunoprecipitations**

For co-immunoprecipitation experiments using fluorescently-tagged ER $\beta$  and NM23-H2 (ER $\beta$ -DsRed and NM23-H2-EGFP), HeLa cells were plated at a density of  $2.5 \times 10^6$  cells on 10 cm diameter tissue culture dishes for 16 hours prior to transfection. Plates were washed twice with phosphate buffered saline (PBS), then incubated with the transfection mix [total of 30  $\mu$ g of ER $\beta$ -DsRed, or both ER $\beta$ -DsRed and nm23-H2-EGFP, and empty DsRed and EGFP as controls (BD Biosciences, Mississauga, ON), 45  $\mu$ g DOTAP:DOPE (1:1 wt; Avanti Polar Lipids Inc, Alabaster, AL) in 75  $\mu$ l Hepes buffered saline (140 mM NaCl, 25 mM Hepes 0.75 mM Na<sub>2</sub>HPO<sub>4</sub> at pH 7.05)] for 6 hours in serum-free DMEM. Subsequently, cells were washed twice with PBS and allowed to grow in 10% FBS DMEM prior to harvest 48 hours post transfection. Lysis in ice cold EBC buffer [50 mM Tris pH 8.0, 120 mM NaCl, 0.5% (v/v) Igepal CA-630 (Sigma) with Complete™ inhibitor (1 tablet per 100 ml; Roche Diagnostics, Laval, QC)] was followed by centrifugation at  $15,000 \times g$  for 15 minutes. For

co-immunoprecipitation experiments using myc-tagged NM23-H2, MCF-7 cells were plated at a density of  $3.5 \times 10^4$  cells in 6-well plates, to reach 90% confluency. Cells were incubated with 2 µg plasmid DNA [pGEM-myc (empty vector, graciously donated by Dr. Heidi McBride, University of Ottawa Heart Institute) or pGEMmyc-NM23-H2, where NM23-H2 cDNA was inserted into the MCS, resulting in an N-terminal myc tag] and 4 µl of Lipofectamine 2000 reagent (Invitrogen), as per the manufacturer's instructions for 6 hours. Plates were washed and replenished with fresh media containing 10% FBS for 48 hours. Protein was collected in an IP buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5% NP-40 in PBS) and centrifuged at high speed for 20 minutes. Two hundred micrograms of cellular protein was incubated with 5 µg of a monoclonal antibody to c-myc (Calbiochem) and 500 µl of IP buffer, and allowed to rotate at 4°C for 2 hours. Protein-G-agarose beads were then added and agitated at 4°C for 30 minutes. The immunoprecipitated material was washed and collected by centrifugation three times in IP buffer to remove unbound material. The final pellet was boiled in SDS loading buffer to remove the agarose beads from the precipitated proteins, and the supernatant was run on an SDS-PAGE gel before the proteins were transferred to a PVDF membrane at 4°C overnight at 20 V. Membranes were then subjected to western blotting using either the anti-c-myc antibody used for the immunoprecipitation (1:500) or a monoclonal antibody to ERβ (1 µg/ml overnight, GeneTex, Texas, USA).

### **Protein Harvesting and Western Blotting**

HeLa, and MCF-7 cell lysates were obtained using a RIPA buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, Complete™ inhibitor in PBS]. Cells were washed twice with PBS, lysed in RIPA buffer, collected using a cell scraper, and placed on ice for 45 minutes. Cell

debris was collected by centrifugation at  $15,000 \times g$  for 20 minutes, and the supernatant containing the cellular protein was isolated. Protein was quantified using Bradford reagent (Sigma). Cell fractionation was carried out as previously described (1). For western blotting, 30  $\mu g$  of whole cell protein was loaded onto a 12.5% SDS-PAGE gel and separated at 90 V using gel electrophoresis. Protein was then transferred to a PVDF membrane (BioRad, Hercules, CA) overnight at 20 V, at 4°C. Membranes were then subjected to western blotting using the following antibodies: a polyclonal anti-DsRed (BD Biosciences); polyclonal ER $\beta$  (Affinity Bioreagents, Golden, CO; at 1 $\mu g/ml$ ); polyclonal anti- NM23-H2 (E. Postel, 1:1000) and a polyclonal anti-cmyc antibody (Calbiochem, Germany; 1:500).

### **Immunohistochemistry**

Cross sections of human breast tissue carcinoma was obtained from the coroner's service at the Vancouver Hospital and Health Sciences Center (Vancouver, Canada) (36). All tissue specimens were immersion fixed with 10% neutral buffered formalin. This post-mortem tissue was harvested within 6 hours of death, and previously we demonstrated preserved protein and mRNA content in these sections (13,19). In addition to hematoxylin and eosin-stained slides, immunolabeling was performed using the following antibodies: a rabbit polyclonal anti-human NM23-H2 (E.H. Postel; dilution 1:200), a rabbit polyclonal anti-ER $\beta$  (1:100, Novus Biologicals, Littleton, CO) and a rabbit anti-ER $\alpha$  (1:100, Santa Cruz) and finally with a biotinylated anti-rabbit IgG (Vector Laboratories) as a secondary antibody. Immunohistochemistry was performed as described previously (4). Briefly, tissue sections were deparaffinized and incubated with 10% horse or goat serum (Vector Laboratories) for 20 minutes to minimize the nonspecific binding of the primary antibody before incubation overnight with one of the primary antibodies in a 4°C moisture chamber. Tissue sections



were then incubated with the appropriate secondary antibody for 30 minutes at RT. To inhibit endogenous peroxidase activity tissue sections were incubated with 3% H<sub>2</sub>O<sub>2</sub> for 30 minutes before incubation with peroxidase-labeled streptavidin (Vector Laboratories) for 30 minutes. Visualization of positive immunolabeling was made possible by the addition of the standard peroxidase enzyme substrate 3,3'-diamino-benzidine tetrahydrochloride (Sigma), which resulted in a brown color reaction product. Hematoxylin was used as the nuclear counterstain. PBS washes were used between each of the aforementioned steps.

### **Confocal Microscopy**

MCF-7 cells were grown on cover slips as described above, and transfected with NM23H2-ECFP construct and an ER $\beta$ -EYFP construct (pECFP-C1 and pEYFP-N1 were obtained from Clontech), using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. After 48 hours, cells were fixed with 4% paraformaldehyde and mounted with Dako Fluorescent mounting media (Dako Cytomation, Mississauga, ON). Cells were visualized with an Olympus FluoView FV1000 confocal microscope (Olympus America Inc, Center Valley, PA) at 100X magnification at 434nm (ECFP) and 514nm (EYFP).

### **ERE Transcriptional Reporter Activation**

MCF-7 cells were transfected with an ERE construct using Superfect (Qiagen, Mississauga, Ont.), as per the manufacturer's instructions. Briefly, a consensus ERE sequence (5'-CGCGTAGGTCACAGTGACCTGATCAAAGTTAATGTAACCTCA-3') was cloned into the multiple cloning site of pGL3-promoter vector (Promega, Madison, WI) and transiently transfected into MCF-7 cells, along with a renilla report vector (pRL-TK, Promega) to control for transfection efficiency. The following constructs were transiently transfected

along with the above ERE-luc reporter construct: an nm23-H2 expression vector (generously donated by Dr. J.L. Parent, University of Sherbrooke, QC), an ER $\beta$  expression vector (pEYFP-N1-ER $\beta$ , generously donated by Dr. A. Al-Madhoun, University of Ottawa, Ontario) and an empty control vector (pcDNA4/His C, Invitrogen) (49). Transfected cells were treated with either serum-supplemented media, or serum-supplemented media plus 100 nmol/L 17 $\beta$ -estradiol for twenty four hours prior to harvesting (10,14). Luciferase activity was measured with a luminometer (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany) using the Dual Luciferase Report Assay system (Promega) as per the manufacturer's instructions. ERE activated transcription (as measured by the firefly luciferase activity, normalized to the renilla luciferase activity) is presented as the relative activity of all samples compared to the control sample (ERE-luc alone with no hormone treatment). All experiments were repeated 3 times (n=6 per group). Please note that although our ultimate goal was to understand the role of NM23-H2 on estrogen-mediated events in vascular cells, it is well recognized that primary human cells are difficult to transfect *in vitro*. Indeed, despite several attempts at transfecting human vascular SMCs with an ERE construct we routinely obtained very low transfection efficiencies regardless of the experimental parameters. Hence, for this reason MCF-7 and HeLa cells were used for the assays involving the ERE transcriptional reporter and MCFs were used for cell migration (see below).

### **Cell Migration Assay**

The migration of MCF-7 cells was assessed using a 48 Well Micro Chemotaxis Chamber (Neuro Probe, Gaithersburg, MD). MCFs transfected with either empty vector (control; pECFP-N1) or an NM23-H2 expression vector (NM23-H2-pECFP) were added to the top well at a density of  $2.0 \times 10^4$  cells per well, in DMEM supplemented with 0.01% FBS.

Media supplemented with either 10% FBS (control) or 10% FBS + 100 nM E2 was added to the bottom wells. Cells were allowed to migrate across a polycarbonate membrane with 8  $\mu$ m pores for 18 hours, at which point the cells were fixed and counted. Data is represented as number of migrated cells per high power field.

### **Statistics**

Values are means  $\pm$  SEM. Comparisons of groups was performed using a One-Way ANOVA. Statistical significance was defined by p value  $<0.05$  and is denoted by an asterisk (\*).

## 3.6 Results

### NM23-H2 associates with ER $\beta$

Using an unbiased approach, we identified previously unreported protein interactions with the unique A/B domain of ER $\beta$  (35). From an initial cDNA library of  $5.3 \times 10^6$  clones only three clones were found to represent true positive interacting proteins (clone B8, C5 and D6) (**Figure 3.1A** and **3.1B**). Clone D6 was identified as nm23-H2, as it shared 98.7% sequence identity with the reported NM23-H2 protein sequence in the open reading frame (Swiss-Prot Accession No. P22392) (**Figure 3.1C**).

To confirm our yeast two hybrid findings, we utilized three *in vitro* assays. First, a MBP assay was performed using both ER $\beta$ -MBP and NM23-H2-intein fusion proteins produced in bacterial systems. With the ER $\beta$ -MBP protein immobilized in a column, cell lysates containing either the intein protein alone or the NM23-H2-intein fusion protein were run through the column. Using an antibody that recognizes the intein domain, western blot analysis of the retained fraction demonstrated minimal background with the intein protein alone and a strong positive interaction with NM23-H2-intein fusion protein (**Figure 3.2A**). Second, HeLa cells were transfected with empty DsRed and EGFP vectors (data not shown), or with either an ER $\beta$ -DsRed fusion construct alone or both ER $\beta$ -DsRed and NM23-H2-EGFP. An anti-DsRed antibody was used to immunoprecipitate proteins bound to ER $\beta$  and isolated proteins were subjected to western blot analysis with an NM23-H2 specific antibody. In HeLa cells transfected with the ER $\beta$  construct, a strong band for endogenous NM23-H2 (17.5 kDa) was observed (**Figure 3.2B**). In the co-transfected HeLa cells, both

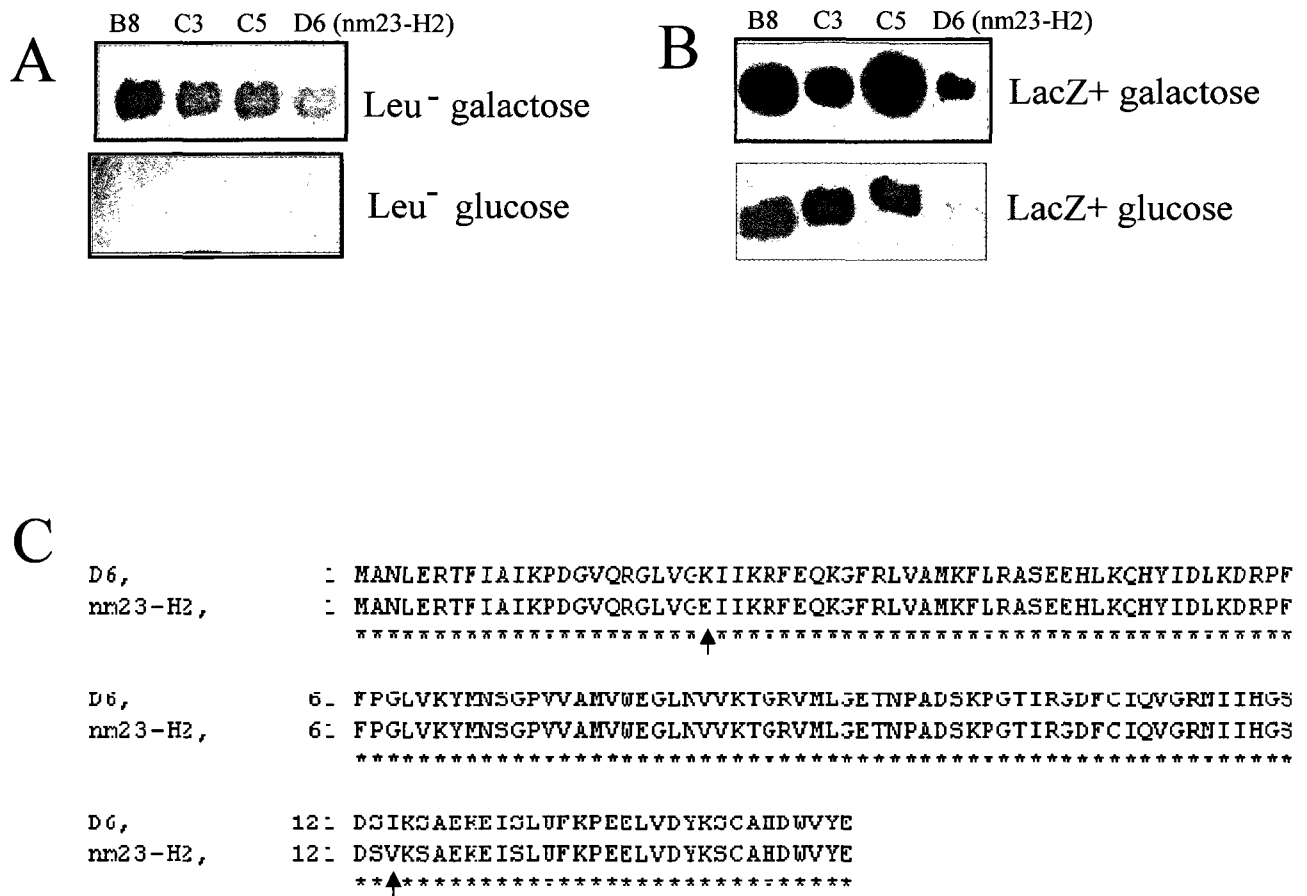
**Figure 3.1: Yeast two hybrid assay using unique A/B region of ER $\beta$  as ‘bait’**

**A)** Yeast were transformed with an ER- $\beta$ -LexA DBD and cDNA clones from the library fused to an activation domain, and selected for growth on galactose (top panel) and glucose (bottom panel) in the absence of leucine (Leu-).

**B)** Yeast were tested for their ability to activate the LacZ gene and turn blue on galactose (top panel) or glucose (bottom panel).

**C)** Positive clone D6 from the screen was sequenced and translated (see Materials and Methods) and identified as NM23-H2. Clone D6 shares 98.7% protein sequence identity with NM23-H2.

**Figure 3.1**



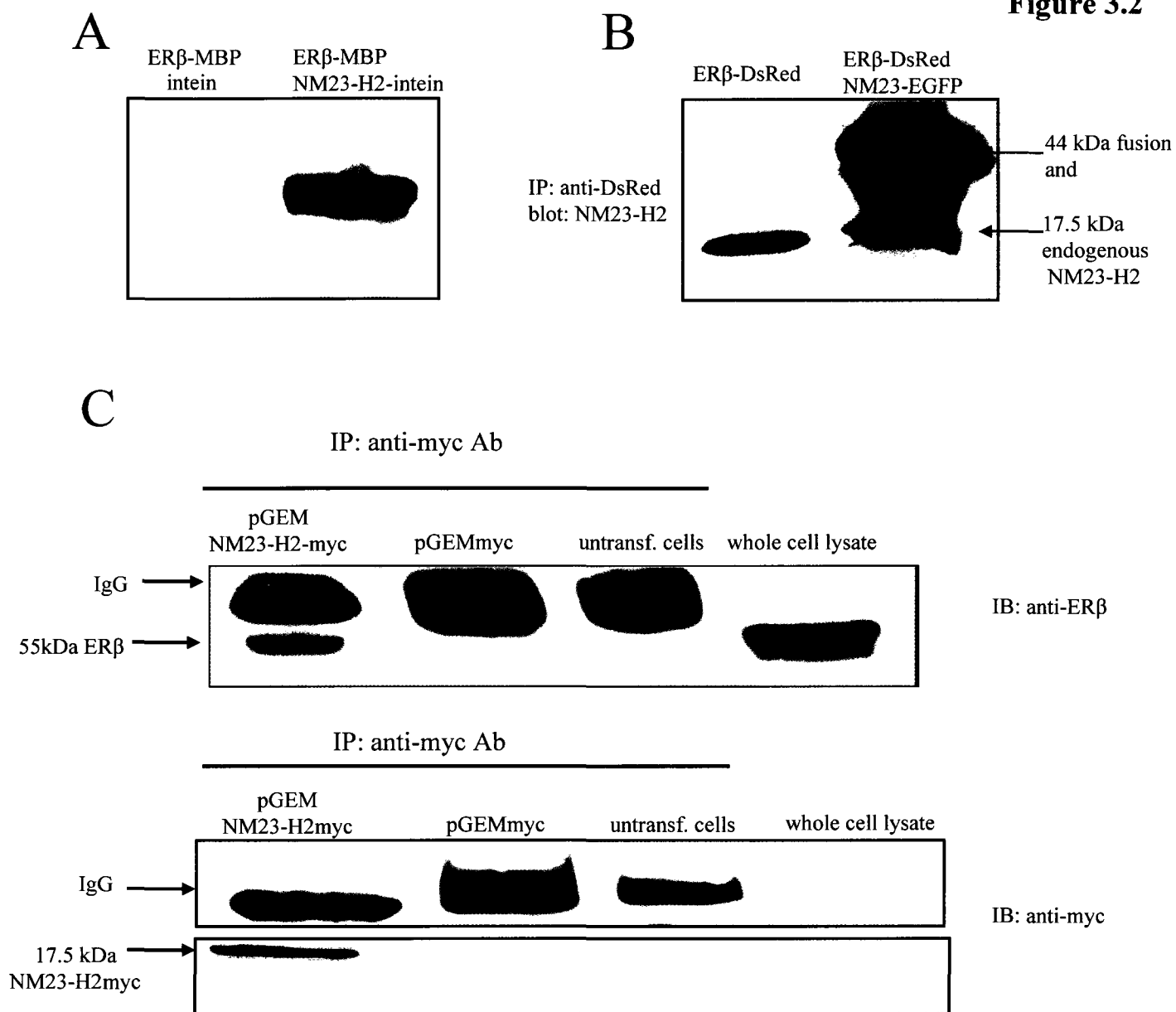
**Figure 3.2: Identification of NM23-H2 as a novel ER $\beta$  associated protein**

**A)** Maltose binding protein assay showing the interaction between the ER $\beta$ -MBP fusion product with intein protein alone (left lane) and NM23-H2-intein fusion product (right lane), using an intein-specific detection antibody.

**B)** A co-immunoprecipitation was performed using HeLa cells transfected with ER $\beta$ -DsRed construct alone (left lane) or ER $\beta$ -DsRed plus nm23-H2-EGFP (right lane). Antibodies to DsRed were used for immunoprecipitation, and antibodies to NM23-H2 were used for detection.

**C)** A co-immunoprecipitation was performed using MCF-7 cells transfected with NM23-H2 tagged with a myc epitope (lane 1) or empty vector (lane 2), and compared to both untransfected cells (lane 3) or whole cell lysate (lane 4). Antibodies to myc were used for the pulldown, while antibodies to ER $\beta$  (top panel) and myc (bottom panel) were used for detection.

**Figure 3.2**





endogenous and fusion NM23-H2 (44 kDa) proteins were immunoprecipitated. Finally, MCF-7 cells were transfected with either an empty myc-tagged vector, or with an NM23-H2-myc tagged vector. An antibody to myc was used for the immunoprecipitation, and antibodies to both myc and ER $\beta$  were used for the detection of bound proteins. As shown in **Figure 3.2C**, NM23-H2-myc was pulled down successfully and a band corresponding to the endogenous ER $\beta$  was observed, while cells transfected with either empty vector or untransfected cells were negative for this interaction. Hence, these assays unequivocally confirm our yeast two hybrid results and demonstrate a novel interaction between NM23-H2 and ER $\beta$ .

#### **NM23-H2 co-localizes with ER $\beta$ but not ER $\alpha$**

Given that NM23-H2 and ER $\beta$  interact *in vitro* both when over-expressed and at endogenous levels, we sought to determine the tissue co-localization of these proteins *in vivo*.

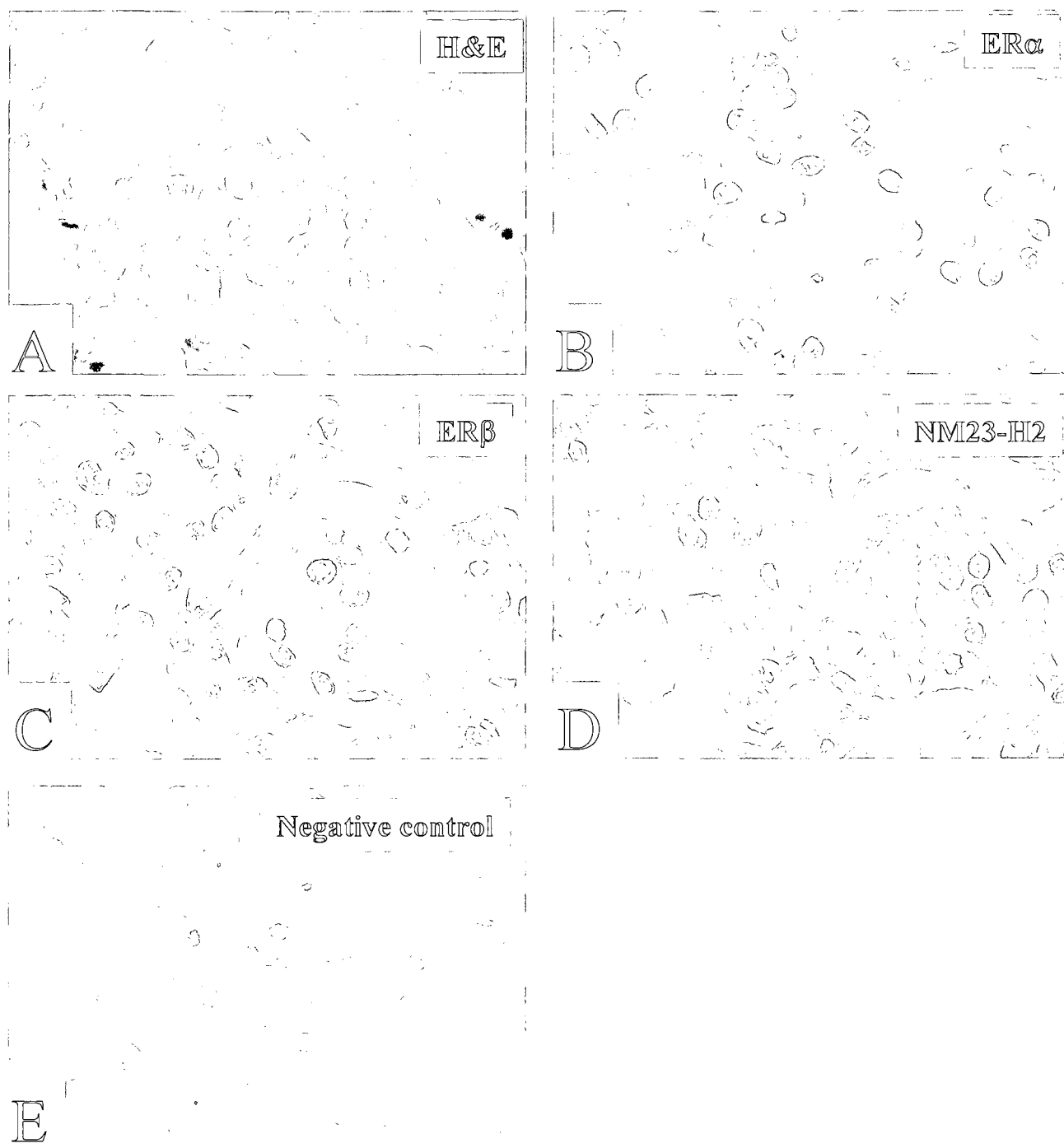
Immunohistochemistry was performed on breast tissue carcinoma sections (**Figure 3.3A**) using antibodies against ER $\alpha$ , ER $\beta$  and NM23-H2. While ER $\alpha$  is clearly found in the nucleus (**Figure 3.3B**), NM23-H2 and ER $\beta$  distinctly immunolocalize in the cytoplasm and not in the nucleus (**Figure 3.3C and 3.3D**). Furthermore, in all tissue types examined (i.e. normal lobular breast tissue and the adjacent connective and nervous tissues), NM23-H2 co-localizes with ER $\beta$ , whereas ER $\alpha$  is found in a distinct cellular compartment (data not shown).

Confocal microscopy was used to further examine the subcellular co-localization of NM23-H2 and ER $\beta$ . MCF-7 cells were transfected with fluorescently-tagged NM23-H2 and ER $\beta$ , before being visualized by excitation of the ECFP and EYFP proteins (**Figure 3.4**). Both

**Figure 3.3: *In vivo* co-localization of NM23-H2 and ER $\beta$**

Breast carcinoma tissue was serially sectioned, stained with hematoxylin and eosin (**A**) and examined for ER $\alpha$  expression (**B**), ER $\beta$  expression (**C**) and NM23-H2 expression (**D**). Non-specific IgG was used as the negative control (**E**).

Figure 3.3



**Figure 3.4: Subcellular co-localization of NM23-H2 and ER $\beta$**

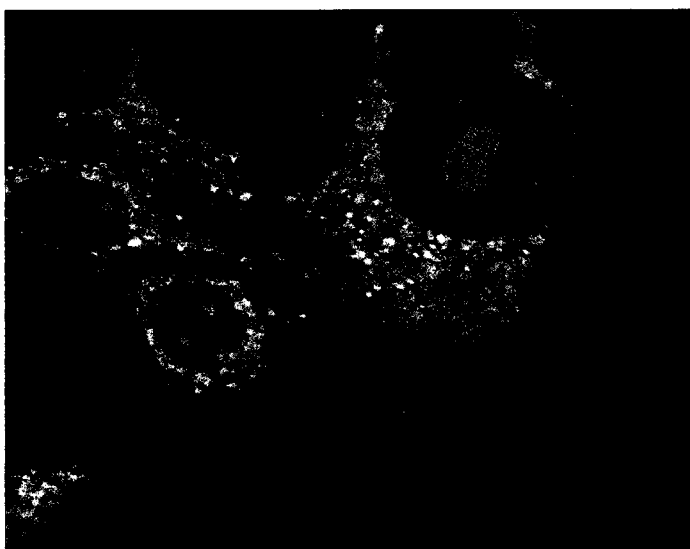
MCF-7 cells were co-transfected with NM23H2-ECFP (top panel, pseudo-coloured red) and ER $\beta$ -EYFP (middle panel, pseudo-coloured green) and visualized using confocal microscopy with excitation at 434nm and 514nm, respectively. The bottom panel shows the merged image, with co-localization of ER $\beta$  and NM23-H2 appearing as yellow colour. Original magnification was 100X.

**Figure 3.4**

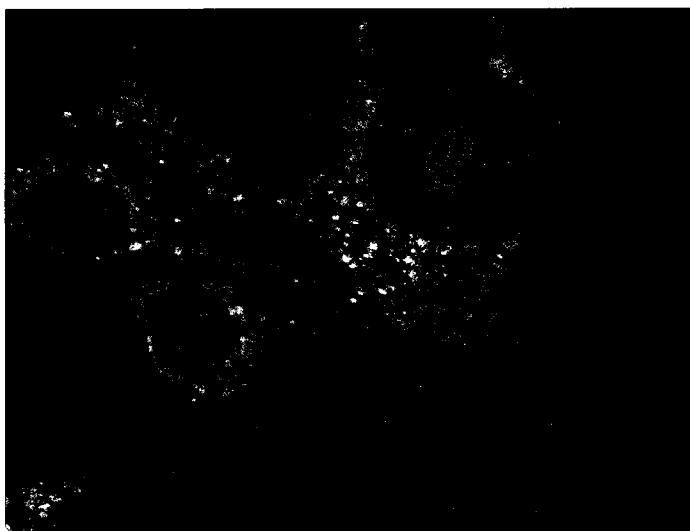
NM23-H2  
(red)



ER $\beta$   
(green)



merged  
(yellow)



proteins were detected in the cytoplasm and shared the same distribution pattern. To a minor degree ER $\beta$  expression was also detected in the nucleus.

### **Effect of NM23-H2 on Estrogen-induced Gene Transcription and Cell Migration**

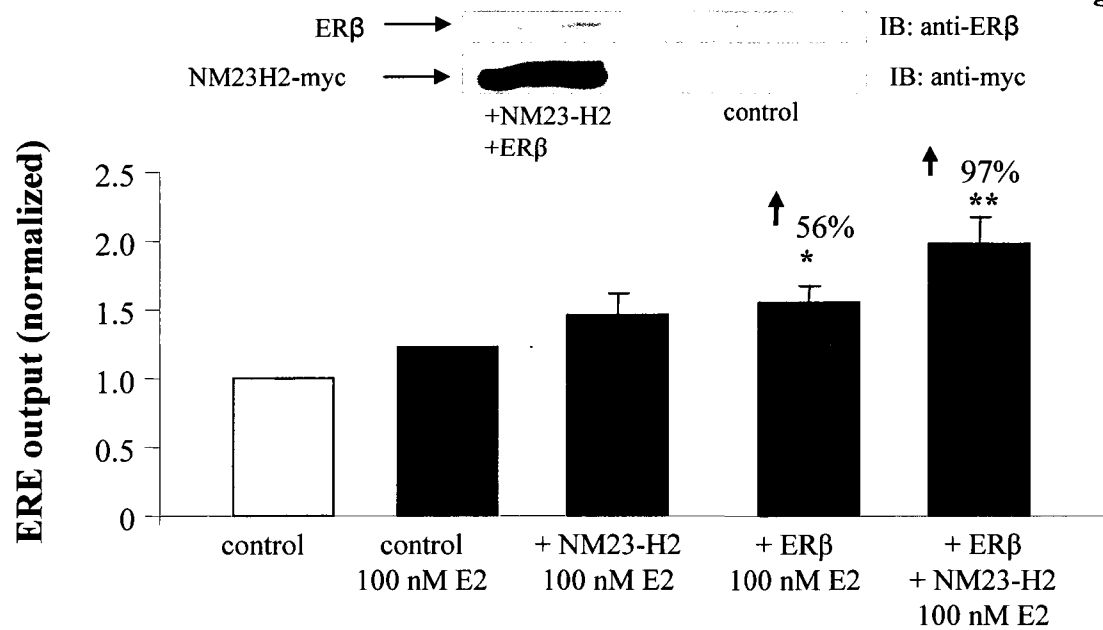
To determine if NM23-H2 is capable of altering estrogen-induced gene expression we employed an ERE-assay. MCF-7 cells and HeLa cells were used for these experiments due to their reproducibly high transfection efficiency, as well as their ability to respond to estrogen. MCF-7s transfected with an ERE upstream of a luciferase reporter gene (ERE-luc), were also co-transfected with an empty control vector (pcDNA4), NM23-H2, ER $\beta$  or both NM23-H2 and ER $\beta$  expression vectors and studied after treatment with either serum alone or serum supplemented with 100 nmol/L 17 $\beta$ -estradiol for 24 hours. There was a 56% increase in ERE output in cells transfected with ERE-luc plus ER $\beta$  compared to cells transfected with ERE-luc alone ( $p < 0.05$ ) (**Figure 3.5A**). However, transfection of ERE-luc with both NM23-H2 and ER $\beta$  resulted in a 97% increase in ERE output ( $p = 0.001$ ). These data indicate that NM23-H2 and ER $\beta$  facilitate estrogen mediated transcription. Cells transfected with ER $\beta$  plus NM23-H2 show a modest 27% increase in ERE activity compared to cells transfected with ER $\beta$  alone. Interestingly, transfection of ER $\beta$  alone results in a positive effect on ERE activity – thereby suggesting the existence of sufficient endogenous NM23-H2 levels. In contrast, transfection of NM23-H2 without ER $\beta$  did not increase ERE output, perhaps because of inadequate endogenous levels of ER $\beta$ . Parallel experiments were performed in HeLa cells, using the same experimental approach as is MCF-7 cells described above. Transfection of NM23-H2 alone resulted in an 89% increase in ERE output compared to controls (**Figure 3.5B**) ( $p < 0.05$ ), suggestive of higher endogenous levels of ER $\beta$  in these cells. When both NM23-H2 and ER $\beta$  were co-transfected, ERE output was increased by

**Figure 3.5: Role of NM23-H2 in Estrogen-induced Gene Transcription and Cell Migration**

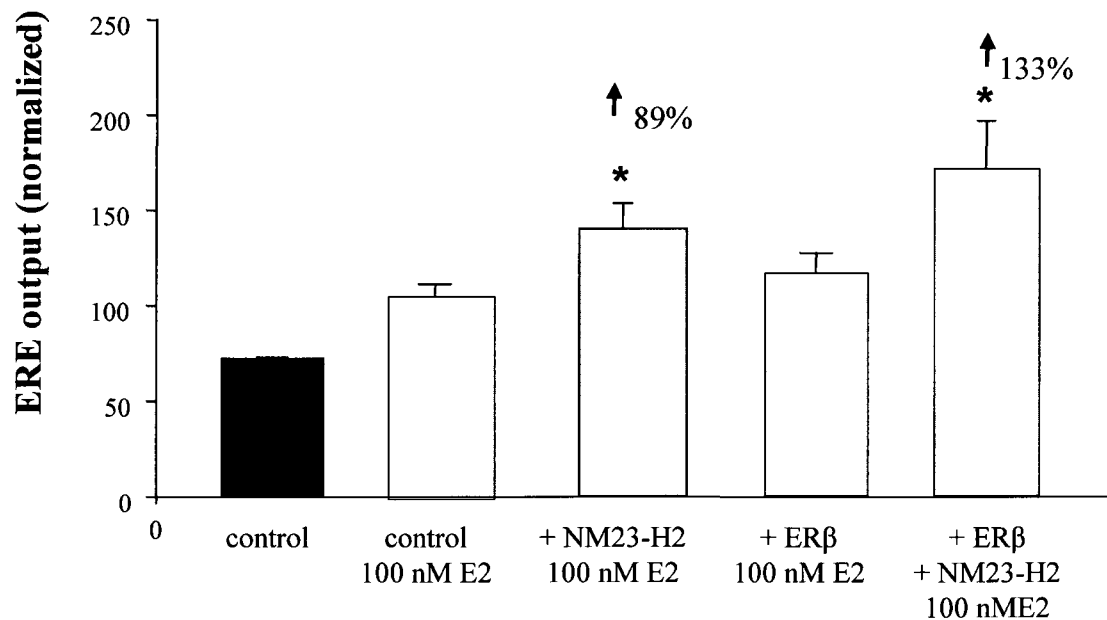
- A).** Measurement of ERE output in MCF-7 cells transiently transfected with an ERE-luciferase reporter construct and a renilla luciferase report construct, as well as the following vectors: NM23-H2 expression vector (+NM23-H2); ER $\beta$  expression vector (+ER $\beta$ ); or both NM23-H2 and ER $\beta$  expression vectors (+NM23-H2 +ER $\beta$ ). All cells were treated with 100 nmol/L 17 $\beta$ -estradiol (black bars) or no hormone (grey bar) and assayed for firefly luciferase activity normalized to renilla luciferase activity in order to control for transfection efficiency. (\*p<0.05, \*\* p=0.001 compared to control; n=6 per group). Also shown is a western blot verifying NM23H2-myc (bottom panel) and ER $\beta$  (top panel) expression in both control cells (empty vectors alone, right column) and cells transfected with expression vectors for NM23-H2 and ER $\beta$  (left column).
- B).** Same experiment as in (A) above, but performed in HeLa cells. (\*p<0.05, compared to control; n=6 per group)
- C).** Western blot of whole cell lysates obtained from MCF-7 cells, HeLa cells and COS-7 cells showing endogenous levels of NM23-H2 and ER $\beta$  in these cells. Blots were probed with antibodies to ER $\beta$  (top panel) and NM23-H2 (bottom panel).

**Figure 3.5**

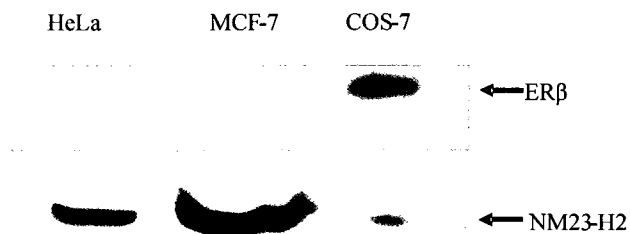
**A**



**B**



**C**





133% compared to controls ( $p < 0.05$ ). However, cells transfected with ER $\beta$  alone had an insignificant increase in ERE activity, suggestive of lower endogenous levels of NM23-H2. The endogenous levels of NM23-H2 and ER $\beta$  were confirmed in both of these cell lines, and demonstrate that indeed HeLa cells express higher levels of endogenous ER $\beta$  compared to MCF-7 cells, whereas MCF-7 cells express higher levels of endogenous NM23-H2 (**Figure 3.5C**). This explains the observation that only when the two proteins are over-expressed in either cell line is a synergistic effect observed.

To test if the activation of ERE transcriptional signaling occurred via translocation of NM23-H2 into the nucleus, MCF-7 cells were fractionated into cytosolic and nuclear compartments and western blotting was performed using antibodies of NM23-H2. After treatment with estrogen, NM23-H2 was visible in the nuclear compartment of these cells, when compared to no treatment (**Figure 3.5D**). This was confirmed under confocal microscopy with cells transfected with NM23-H2-ECFP (pseudo-coloured green) and ER $\beta$ -EYFP (pseudo-coloured red) under estrogen treatment. NM23-H2 is therefore capable of moving to the nucleus following treatment with 17 $\beta$ -estradiol, where it possibly acts as a co-activator of ERE-transcriptional activation. These results agree with those previously observed by our lab in human coronary smooth muscle cells (48).

Given our interest in atherosclerosis and that atherogenesis is a complex process that involves many events, including the migration of a variety of cell types (e.g. macrophages, SMCs), we hypothesized that the previously reported anti-metastatic properties of NM23-H2 might affect cellular migration (18). Moreover, in that estrogen can also inhibit cell migration (14) we postulated that NM23-H2 and estrogens may have synergistic anti-

migratory effects. To assess the effects of NM23-H2 on cell migration, MCF-7 cells were transfected with either empty vector (control) or an NM23-H2 expression vector (NM23-H2-ECFP) and migration was measured using a modified Boyden chamber (**Figure 3.5E**). Compared to empty vector alone, cell migration was reduced by 15.1% and 21.7% when cells were transfected with NM23-H2 or treated with estrogen, respectively (empty vector  $24.4 \pm 1.0$  ; NM23-H2 transfected cells  $20.7 \pm 0.8$ ; empty vector plus 100 nmol/L estrogen  $19.1 \pm 0.5$ ;  $p \leq 0.001$ ). When cells were both transfected with NM23-H2 and treated with estrogen cell migration was reduced by 34.1% compared to controls ( $16.1 \pm 0.8$ ;  $p \leq 0.001$ ). These data demonstrate that NM23-H2 is capable of reducing migration of MCF-7 cells, and this effect is enhanced in the presence of estrogen.

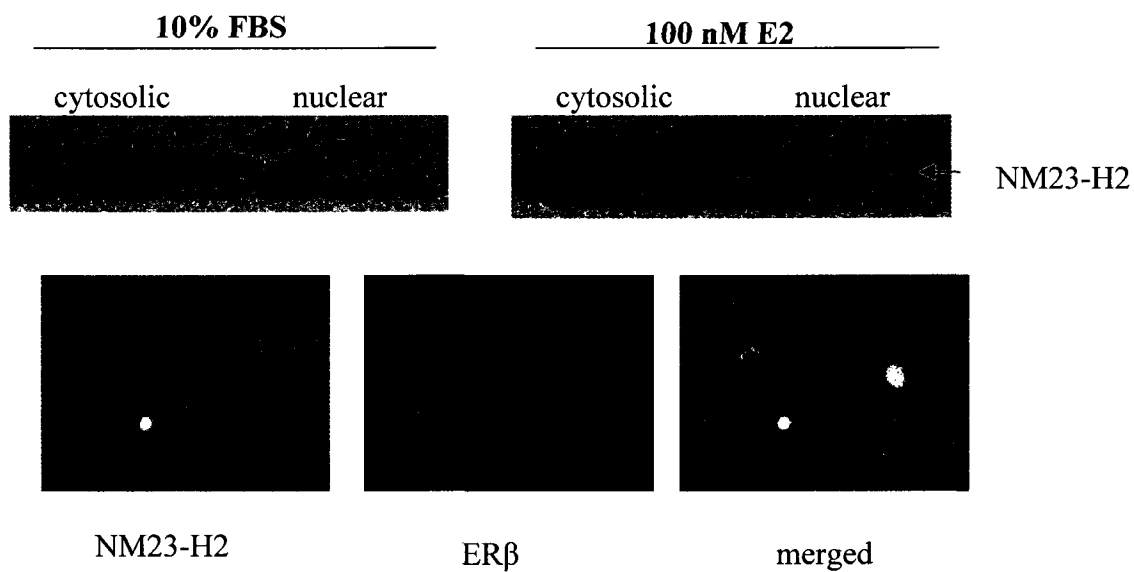
**Figure 3.5: Role of NM23-H2 in Estrogen-induced Gene Transcription and Cell Migration**

**D).** Fractionation of the cytosolic and nuclear compartments of MCF-7 cells under control conditions (10%FBS; left panel) and treatment with 17 $\beta$ -estradiol (100nM E2; right panel) and probed with an antibody to NM23-H2. Bottom shows fluorescent micrographs of MCF-7 cells in culture stimulated with 100 nM E2 and immunolabeled using antibodies to NM23-H2 (green) and ER $\beta$  (red).

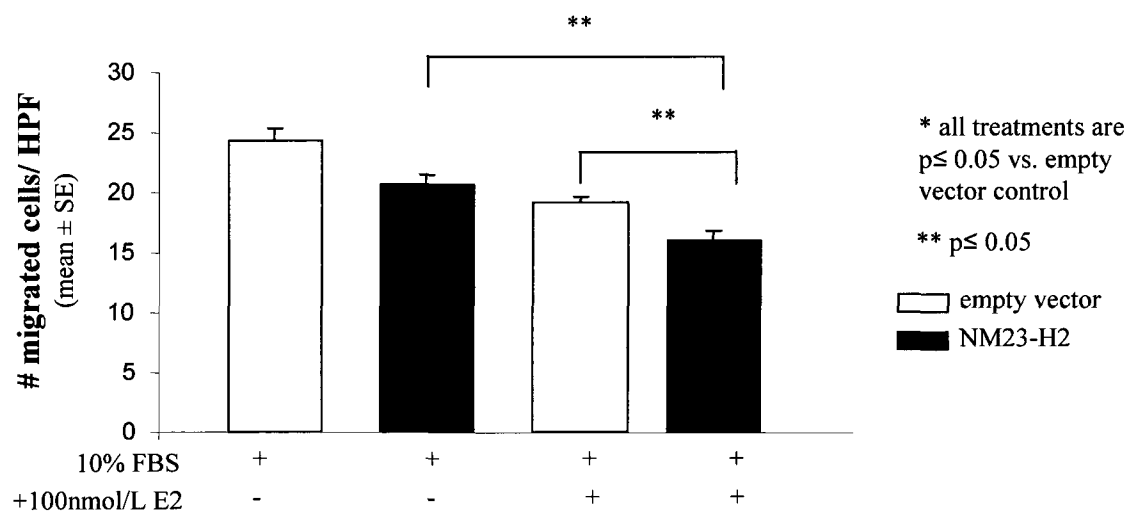
**E).** Quantitation of MCF-7 cell migration using a modified Boyden chamber. Cells were transfected with empty vector (open bars) or an NM23-H2 expression vector (black bars) before being treated with media supplemented with either 10% FBS or 10% FBS with 100 nmol/L 17 $\beta$ -estradiol. (\*p $\leq$ 0.001 and \*\*p $\leq$ 0.05 compared to empty vector alone). HPF= high power field.

**Figure 3.5**

**D**



**E**



### 3.7 Discussion

As we are interested in the potential role of estrogens in vascular disease, we sought to identify and characterize proteins that might associate and modulate the actions of ER $\beta$ , the ER isoform that is of emerging importance in the development of vascular disease (6,27,28,33). By means of a yeast two hybrid screen, we discovered the novel interaction of the unique A/B domain of ER $\beta$  with NM23-H2 and confirmed this interaction using three separate *in vitro* assays. NM23-H2 and ER $\beta$  co-localized and interacted both when endogenously expressed, as well as when over-expressed *in vitro*. As demonstrated using immunolabeling, NM23-H2 and ER $\beta$  readily co-localized in breast tissue – however, ER $\alpha$  did not co-localize with either NM23-H2 or ER $\beta$ . Moreover, using an *in vitro* ERE reporter assay, estrogen mediated transcription increased by 97% when NM23-H2 and ER $\beta$  were over-expressed. Finally, over-expression of NM23-H2 was synergistic with 17 $\beta$ -estradiol treatment in reducing the migration of MCF-7 cells *in vitro*. Recently, we demonstrated that NM23-H2 expression is attenuated with the development of vascular disease (e.g. atherosclerosis). While we recognize that, for technical reasons, these studies were not performed in vascular cells and hence there are limitations to extrapolating the data, it is attractive to postulate that loss of NM23-H2 expression with the development of vascular disease may result in faulty ER $\beta$  regulation and have specific implications for the role of estrogen in the vessel wall.

The human nm23 gene family consists of eight members (nm23-H1 through –H8), with nm23-H1 and nm23-H2 both localized on chromosome 17q21.3 (3,54) and having gene

products that share 88% identity. nm23 genes encode for NDP kinases (NDPKs) are multifunctional proteins that carry out a spectrum of regulatory functions (for a review please see (20) that extend beyond the simple transfer of a  $\gamma$ -phosphate between nucleoside tri- and di-phosphates (44). The NDPKs are 17–20 kDa proteins that associate *in vivo* to form homo- or heterohexamers and interact with other proteins, some of which are involved in cancer progression (29). For example, over-expression of NM23-H1 in several metastatic cell lines results in reduced metastatic potential both *in vitro* and *in vivo* (26,42). NM23 proteins also bind and cleave DNA (45) as well as regulate important promoters, including those for *c-myc* (45,46) and PDGF-A (32). NDPKs can locally modulate the GTP pool and in doing so influence signal transduction (40, 21). Abnormal wing discs (*awd*), the *Drosophila* ortholog of Nm23-H1 and Nm23-H2 that is required for proper differentiation of tissues of epithelial origin (9,50,53), is also proposed to be an activator of the GTPase dynamin that facilitates endocytosis of growth factor receptors - thereby attenuating growth factor signaling (7,23,43). Recent studies implicate NM23 proteins as partners of nuclear proteins associated with cell proliferation (30), or centrosome-associated proteins (51). Finally, *in vitro* analyses demonstrating that NM23-H2 is associated with human telomeres and the RNA component of telomerase (39).

In this study we show that NM23-H2 had two important influences on the cellular actions of estrogens. First, there was a modest facilitation (97% increase) in estrogen mediated gene transcription when NM23-H2 was over-expressed. This result is particularly interesting since our immunocytochemistry studies localized NM23-H2 to the cytoplasm and not the nucleus. While the bulk of the protein seems to be in the cytoplasm, nuclear localization of NM23-H2 in cultured human cells, including vascular smooth muscle cells is well documented (22,48).

The significance of the cytoplasmic interaction of NM23-H2 and ER $\beta$  is unclear. As NM23-H2 increases estrogen-mediated ERE transcription, NM23-H2 may facilitate the nuclear translocation of ER $\beta$  in a manner yet to be determined. Moreover, as described above, NM23-H2 is involved in several other well-documented nuclear activities (e.g., DNA cleavage, transcriptional regulation of c-myc and PDGF-A promoters). The second notable effect of NM23-H2 was its synergy with 17- $\beta$  estradiol in reducing cell migration. This result is not surprising, in that NM23 isoforms can form complexes with proteins involved in cell migration, such as Tiam1, the specific GDP/GTP exchange factor of Rac1 (41). Whether the reduction of migration is somehow related to these protein-protein interactions or perhaps other means (e.g., sequestration of receptors for growth factors that might induce cell migration (7,23,43) or the interaction with proteins involved in cell movement and adhesion (11,12) is unclear.

In summary, NM23-H2 associates with and can act as a co-activator of ER $\beta$ . Moreover, NM23-H2 has a modest role in modulating estrogen-induced gene transcription and acts synergistically with 17 $\beta$ -estradiol to reduce cell migration. Given that NM23-H2 expression is reduced with the progression of vascular disease and in the presence of inflammation, it is intriguing to speculate that NM23-H2 plays an important functional role in vessel wall homeostasis that is directly related to its interaction with ER $\beta$ . If NM23-H2 expression is down-regulated during the inflammatory stages of vascular disease, and hormonal replacement therapy is initiated, could this result in unregulated estrogen mediated transcription and cell migration that leads to adverse consequences in the vessel wall? Alternatively, NM23-H2 may also have ER $\beta$  independent vascular effects. For example, recent studies indicate that NM23 plays an important role in reducing the ill-effects of

reactive oxygen species – factors that are known to be an integral part of the multi-step process of atherogenesis (8). Both NDP kinases can protect cells from oxidative stress-induced death (2,37). Our ongoing studies with mice lacking functional NM23 and/or ERs will help determine the exact functional consequences of the NM23-H2/ER $\beta$  interaction in vessel wall.



### **3.8 Acknowledgments**

This work was supported by a grant-in-aid (#T5543) to EOB. During the course of these studies KR held a Master's Studentship Award from the Heart and Stroke Foundation of Ontario (HSFO) and a Doctoral Research award from the Ontario Women's Health Council/Canadian Institutes of Health Research. EHP is supported by an NIH/NCI grant (#RO1 CA076496). EOB holds a Research Chair that is jointly funded by the Canadian Institutes of Health Research and Medtronic.

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#### **4.0 MANUSCRIPT # 3:**

##### **Extracellular Release of the Atheroprotective Heat Shock Protein 27 is Mediated by Estrogen and Competitively Inhibits acLDL Binding to Scavenger Receptor-A**

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##### *Circulation Research*

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#### **4.1 Significance of this Manuscript**

This study provides new evidence that HSP27, a multi-faceted stress protein, can protect against atherosclerosis. Moreover, we show that this effect appears to be dependent upon estrogen, as only female mice over-expressing HSP27 are protected from disease, whereas males are not. Interestingly, there is a marked inverse correlation between the levels of HSP27 in the serum (i.e. extracellular space) and degree of atherosclerosis- the higher the levels, the more protection. We also discovered a receptor for HSP27 on the surface of macrophages- the scavenger receptor-A. When HSP27 binds SR-A, atherogenic lipid uptake is reduced and inflammation is attenuated. This work shows for the first time that extracellular HSP27 may act to attenuate atherosclerosis by binding SR-A and preventing foam cell formation- a significant discovery in our understanding of the pathogenesis of atherosclerosis and how HSP27 may one day be used as a therapy for the prevention of disease.

## **4.2 Author contributions:**

With the exception of the lesion analysis performed in Figure 1, I designed and conducted most experiments in this paper, with the technical assistance of the co-authors. I wrote the majority of the manuscript with assistance and guidance from my supervisor, and was responsible for the reviews to both the written manuscript and the experimental additions throughout the publication process.



### 4.3 Abstract

We recently identified heat shock protein 27 (HSP27) as an estrogen receptor beta (ER $\beta$ ) associated protein and noted its role as a biomarker for atherosclerosis. The current study tests the hypothesis that HSP27 is protective against the development of atherosclerosis. HSP27 over-expressing (HSP27<sup>o/e</sup>) mice were crossed to apoE<sup>-/-</sup> mice that develop atherosclerosis when fed a high-fat diet. Aortic en-face analysis demonstrated a 35% reduction ( $p \leq 0.001$ ) in atherosclerotic lesion area in apoE<sup>-/-</sup>HSP27<sup>o/e</sup> mice compared to apoE<sup>-/-</sup> mice, but primarily in females. Serum HSP27 levels were >10-fold higher in female apoE<sup>-/-</sup> HSP27<sup>o/e</sup> mice compared to males, and there was a remarkable inverse correlation between circulating HSP27 levels and lesion area in both male and female mice ( $r^2=0.78$ ,  $p \leq 0.001$ ). Mechanistic *in vitro* studies showed up-regulated HSP27 expression and secretion in macrophages treated with estrogen and/or acLDL. Moreover, exogenous HSP27 added to culture media inhibited macrophage acLDL uptake and competed for the scavenger receptor –A (SR-A) – an effect that was abolished with the SR-A competitive ligand fucoidan and absent in macrophages from SR-A<sup>-/-</sup> mice. Furthermore, extracellular HSP27 decreased acLDL-induced release of the pro-inflammatory cytokine IL-1 $\beta$  and increased the release of the anti-inflammatory cytokine IL-10. HSP27 is atheroprotective, perhaps due to its ability to compete for the uptake of atherogenic lipids and/or attenuate inflammation.

#### 4.4 Introduction

Studies from our laboratory as well as others reveal that estrogen receptor  $\beta$  (ER $\beta$ ) may play a key role in vascular homeostasis (22). For example, ER $\beta$  mRNA and protein expression are up-regulated after vascular injury in male arteries (17,20). Moreover, ER $\beta$  is the predominant receptor expressed in the intima, media and adventitia in male arteries, and unlike ER $\alpha$ , its expression correlates with the degree of calcification – a marker of severe atherosclerosis (6,19). Recently we discovered that heat shock protein 27 (HSP27) interacts with estrogen receptor  $\beta$  (ER $\beta$ ) to reduce estrogen-mediated transcriptional signaling, as reflected by the results of an estrogen response element (ERE) activity assay. Moreover, we showed that HSP27 expression diminishes with the progression of atherosclerosis (22). Hence, this loss of HSP27 acting as a co-repressor on estrogen signaling may result in the untoward expression of genes associated with atherogenesis.

Heat shock proteins are involved in a wide variety of processes, both physiological and pathological (34,35). Heat shock protein 27 (HSP27) is a member of the small (15 to 30 kDa) heat shock protein family. Principally described as an intracellular chaperone, HSP27 is capable of binding and stabilizing the actin cytoskeleton in response to stress (see review by Ciocca *et al.*, 7). In addition, HSP27 can bind cytochrome c and prevent downstream caspase activation, thereby making it a potent anti-apoptotic protein (8,33). Hence, given the interaction between HSP27 and ER $\beta$ , we sought to further elucidate the vascular role of HSP27, and the role of estrogens in this process. Our laboratory as well as others recently proposed that HSP27 is a biomarker for atherosclerosis, with vascular HSP27 expression diminishing with the progression of disease (21,22,24). Indeed, we demonstrated that

HSP27 tissue expression diminishes in human coronary arteries of young individuals (mean age: 27 years) as atherosclerosis stage increases (22). Separate proteomic studies discovered decreased HSP27 levels secreted from human carotid endarterectomy plaques vs. normal (disease free) arteries (21) and preserved HSP27 expression in cardiac biopsy specimens (primarily blood vessels) correlated with freedom from cardiac allograft vasculopathy (9,25). Moreover, HSP27 is present in the serum of individuals free of atherosclerosis (21,24) and presumably acts in a yet to be defined “atheroprotective” manner. In other tissues (e.g. nerve, gastromucosal and myocardium) HSP27 is protective against the sequelae of acute injury/stress, perhaps via anti-apoptotic mechanisms (1,15,18,27,36).

Hence, the central postulate of this study is that HSP27 is protective against atherogenesis, and in order to address this claim we determined if over-expression of human HSP27 in atherosclerosis-prone apoE<sup>-/-</sup> mice was protective against development of disease. We now report for the first time how extracellular HSP27 may have a potent atheroprotective effect that is modulated by estrogens and high-fat diet, and involves interaction with the scavenger receptor A with anti-inflammatory effects.

## 4.5 Materials and Methods

### Murine Atherosclerosis Model

All experimental procedures involving laboratory animals were performed with approval from the Animal Care and Use Committee of the University of Ottawa. Transgenic mice over-expressing human HSP27 (HSP27<sup>o/e</sup>) (under the control of a chicken  $\beta$ -actin promoter and a CMV enhancer element) on a C57BL10/CBA background were provided by Imperial College London, and were maintained by continuously backcrossing with C57BL10/CBA mice.(2) ApoE<sup>-/-</sup> mice with the C57BL/6 genetic background were purchased from the Jackson Laboratory (Bar Harbor, Maine). HSP27<sup>o/e</sup> females were crossed with apoE<sup>-/-</sup> males to generate apoE<sup>+/-</sup>HSP27<sup>o/e</sup> mice, which were crossed again to apoE<sup>-/-</sup> mice to generate HSP27<sup>o/e</sup> apoE<sup>-/-</sup> (n=6 males and n=9 females) and apoE<sup>-/-</sup> (n=6 males and n=9 females) littermates. Genomic DNA was extracted from the tail tips for genotyping apoE and HSP27 as described previously.(3) Mice were fed a normal chow diet until 6 weeks of age, wherein they received high-fat diet (1.25% cholesterol, 15.8% fat; Harlan Teklad, Madison, WI) for 4 weeks. The mice were then euthanized and blood samples were collected to determine serum levels of total cholesterol using an enzymatic assay kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan). After perfusing with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS via the left ventricle, the heart and aorta were removed and immersed in 4% PFA/PBS at 4°C overnight. Adventitial tissue was removed, and the aorta was opened longitudinally and pinned onto a black wax surface using microneedles. Lipid-rich intraluminal lesions were stained with oil red O. Serial images of the submerged vessels were captured with a video camera. En face atherosclerotic lesions of aortic arch were

analyzed by two observers using Image-Pro software (Media Cybernetics, Silver Spring, MD) to calculate the total and atherosclerotic lesion areas. The amount of aortic arch lesion formation in each animal was measured as percent lesion area per total area of the aortic arch. The descending aorta was lesion-free in all groups. In order to measure the serum levels of HSP27, blood samples were collected at baseline (before the commencement of high-fat diet), and after 2 and 4 weeks of high-fat diet, and stored at -80°C until further use.

### **HSP27 Enzyme Linked Immunosorbent Assay**

Plasma levels of HSP27 were measured using an ELISA kit specific to human HSP27 (QIA119, Calbiochem, San Diego, CA). A standard curve of known amounts of HSP27 was constructed with each assay. Serum from HSP27<sup>o/e</sup> apoE<sup>-/-</sup> and apoE<sup>-/-</sup> mice was diluted 1:10 in dilution buffer, and assayed according to the manufacturers' protocol. This assay was found to have no cross-reactivity with mouse HSP25.

### **Cell Culture-Macrophage Cell Lines and Reagents**

After sacrifice, mouse peritoneal macrophages were immediately obtained from peritoneal lavage with 9 ml sterile PBS. Cells were centrifuged and washed twice with PBS and resuspended in culture medium before counting and plating in 24-well plates. Scavenger receptor-A knock out mice (SR-A KO) were a generous gift from Dr. Yves Marcel (University of Ottawa Heart Institute), and were used for collection of peritoneal macrophages at 12 weeks of age. Peritoneal macrophages and J774 mouse macrophages (American Type Culture Collection, ATCC; Manassas, Va) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, Wisent, Saint-Jean-Baptiste de Rouville, QC), penicillin-streptomycin

(Invitrogen), and fungizone (Invitrogen). U937 human macrophages (ATCC) were cultured in RPMI 1640 (Invitrogen) supplemented with sodium pyruvate and glutamine (Invitrogen), penicillin-streptomycin and 10%FBS. Differentiation was induced with 100 nM phorbol myristate acetate (PMA) for 72 hours before any treatment was initiated. Acetylated low-density lipoprotein (acLDL) was obtained from Intracel (Frederick, MD), and DiI-labeled acLDL was obtained from Invitrogen. Fucoidan and estrogen (17 $\beta$ -estradiol) were obtained from Sigma-Aldrich (Oakville, ON). LysoTracker® Red was obtained from Invitrogen. Recombinant low-endotoxin human HSP27 was purchased from Stressgen (Ann Arbor, MI, catalog #ESP-715) and contained less than 50 EU of endotoxin per mg of protein. Peritoneal macrophages harvested from apoE<sup>-/-</sup> and HSP27<sup>oe</sup>apoE<sup>-/-</sup> mice were plated for 2 hours and fixed using 4% PFA, and subjected to staining with Oil Red O, after which slides were mounted and examined using a light microscope. A total of 12 images were obtained from each treatment group, each containing an average of 10-15 cells per image. Semi-quantitative analysis was performed as follows: a cell that stained deep red was considered “lipid laden” compared to those staining light brown (or not significantly higher than background) which were considered “normal”. Each cell type was counted and expressed as a percentage of total cell number, by two independent reviewers blinded to the experimental conditions.

### **Conditioned Media Collection**

An equal number of cells (1X10<sup>6</sup> per ml) were plated in 24-well plates in an equal volume of media. Following treatment, supernatants were spun at 1,000  $\times$  g for 5 minutes to remove any non-adherent or dead cells. Conditioned media samples were analyzed for cell viability and membrane integrity using the CytoTox 96® Non-Radioactive Cytotoxicity Assay

(Promega, San Luis Obispo, CA). LDH release (a measure of cell injury) did not differ between any of the treatments, and was not significantly different than cell culture media alone (without cells). An equal volume (40  $\mu$ l) of media from each sample was subjected to Western blotting.

### **Transfections**

J774 cells were chosen for all transfection experiments due to their relatively high transfection efficiency compared to human U937s. Cells were transfected using Fugene HD transfection reagent (Roche, Laval, QC) as per the manufacturer's instructions. Cells were grown to 90% confluence on coverslips and transfected at a ratio of 2:3 (DNA:reagent) with HSP27-ECFP (pECFP-C1 was obtained from Clontech) for 24 hours before any treatment was initiated.

### **Western Blotting**

Cell lysates were obtained using a RIPA buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, Complete™ inhibitor in PBS]. Cells were washed twice with PBS, lysed in RIPA buffer, collected using a cell scraper, and placed on ice for 45 minutes. Cell debris was collected by centrifugation at  $15,000 \times g$  for 20 minutes, and the supernatant containing the cellular protein was isolated. Protein was quantified using Bradford reagent (Sigma). For western blotting, 50  $\mu$ g of whole cell protein or 40  $\mu$ l of conditioned media were loaded onto a 10% SDS-PAGE gel and separated at 120 V using gel electrophoresis. Protein was then transferred to a PVDF membrane (BioRad, Hercules, CA) for 2 hours at 60 V. Membranes were then subjected to western blotting using the following antibodies: monoclonal anti-

HSP27 (Chemicon, 1:200); polyclonal anti-SR-A (Chemicon, 1:2000); polyclonal anti-IL-1 $\beta$  (R&D, 1:500); polyclonal anti-IL-10 (Santa Cruz, 1:200).

### **Immunolabeling**

J774 cells treated with recombinant HSP27 (5  $\mu$ g/ml)(1) with or without fucoidan (10  $\mu$ g/ml) or U937 cells treated with or without E2 were fixed with BD Cytotfix (BD Biosciences, Mississauga, ON) and blocked with 2% bovine serum albumin (BSA) for 2 hours. Antibodies against human HSP27 (monoclonal, Chemicon, 1:200), LAMP1 (polyclonal, Santa Cruz, 1:1000) and scavenger receptor A (polyclonal, Serotec or R&D, 1:200) were incubated overnight at 4°C. Visualization of substrates was done using secondary antibodies conjugated to fluorescein and Texas Red. Negative controls included incubation with control IgG and secondary antibody alone.

### **Cross-linking and Immunoprecipitations**

J774 cells were transfected with HSP27-ECFP as described above, and treated with 100 $\mu$ g/ml acLDL for 24 hours. Conditioned media from these cells was applied to naïve (untransfected) macrophages for 2h at 4°C to allow HSP27 to bind the cell surface. Cross-linking was performed using 3, 3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP, Pierce, Rockford, IL) as per the manufacturer's instructions. Cells were harvested in immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5% NP-40 in PBS) and 100  $\mu$ g of total protein was immunoprecipitated with 5  $\mu$ g of anti-ECFP antibodies (A.V. peptide, BD Biosciences) overnight at 4°C with shaking. Protein-G-agarose was added for 4 hours, following which cells were centrifuged at 14,000  $\times$  g and washed four times in IP



buffer. Finally, IP pellets were resuspended in SDS-PAGE loading buffer with  $\beta$ -mercaptoethanol to reverse the cross-linking and subjected to western blotting.

### **Confocal Microscopy**

Cells were grown on cover slips, and transfected as described above. Cells were fixed with BD Cytofix as directed by the manufacturer and mounted with Dako Fluorescent mounting media (Dako Cytomation, Mississauga, ON). Cells were visualized with an Olympus FluoView FV1000 confocal microscope (Olympus America Inc, Center Valley, PA) at 100X magnification, using sequential scanning of each fluorophore to reduce any potential non-specific excitation of the different fluorophores. Additionally, cells were visualized after staining with only one fluorophore and no bleed-through into the opposite channel was observed.

### **Fluorescent acLDL uptake**

Cells were treated with DiI-acLDL (5 $\mu$ g/ml) for 4 hours at 37°C, in the presence of recombinant HSP27 (5 $\mu$ g/ml) and/or fucoidan (10 $\mu$ g/ml). BSA was used as a negative control in some experiments. Cells were harvested by gentle cell scraping in 100 $\mu$ l of PBS. Each sample was analyzed for acLDL fluorescence on a BMG PolarStar plate reader (excitation: 510nm, emission: 570nm) and normalized to total cell number using the Vi-Cell cell counter (Beckman Coulter, Mississauga, ON).

### **Cell Adhesion Assay**

Peritoneal macrophages were harvested from apoE<sup>-/-</sup> mice and apoE<sup>-/-</sup>HSP27<sup>o/e</sup> mice as described above. Cells (1.6 x 10<sup>5</sup>) were plated on type I collagen-coated 35 mm-dishes in

10% FBS DMEM. After 2 hours of incubation at 37°C, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 1 hr at 4°C. Cell nuclei were stained with Hoechst 33258 for 10 min at RT. Photos were taken under fluorescence microscopy at 10 x magnification, and the number of attached cells was manually counted per high power field (HPF).

### **Cell Migration Assay**

Peritoneal macrophages were harvested from apoE<sup>-/-</sup> mice and apoE<sup>-/-</sup>hHSP27<sup>o/e</sup> mice as described above. Cells ( $5.0 \times 10^4$ ) were plated in the top chamber of a CytoSelect™ 96-Well Cell Migration Assay (Cell Biolabs, San Diego, CA) in 0.1% FBS-DMEM. The bottom chamber contained 10% FBS-DMEM as a chemoattractant. The migration chamber was incubated at 37°C overnight, and migrated cells were quantified using a fluorescent dye (as per the manufacturer's instructions) and the BMG PolarStar plate reader (excitation: 488nm, emission: 520nm).

### **Statistical Analysis**

All data represent mean  $\pm$  SEM, except as specifically stated. Each experiment was conducted at least 3 times. Statistical analysis was performed with one-way ANOVA by using SigmaStat 3.5 software. Correlation analysis was performed with linear regression by using SigmaPlot 10.0 software. Differences were considered significant at p-value <0.05 and is denoted by an asterisk (\*) or a pound sign (#).

## 4.6 Results

### HSP27 Over-expression Protects Against Atherogenesis in Female Mice

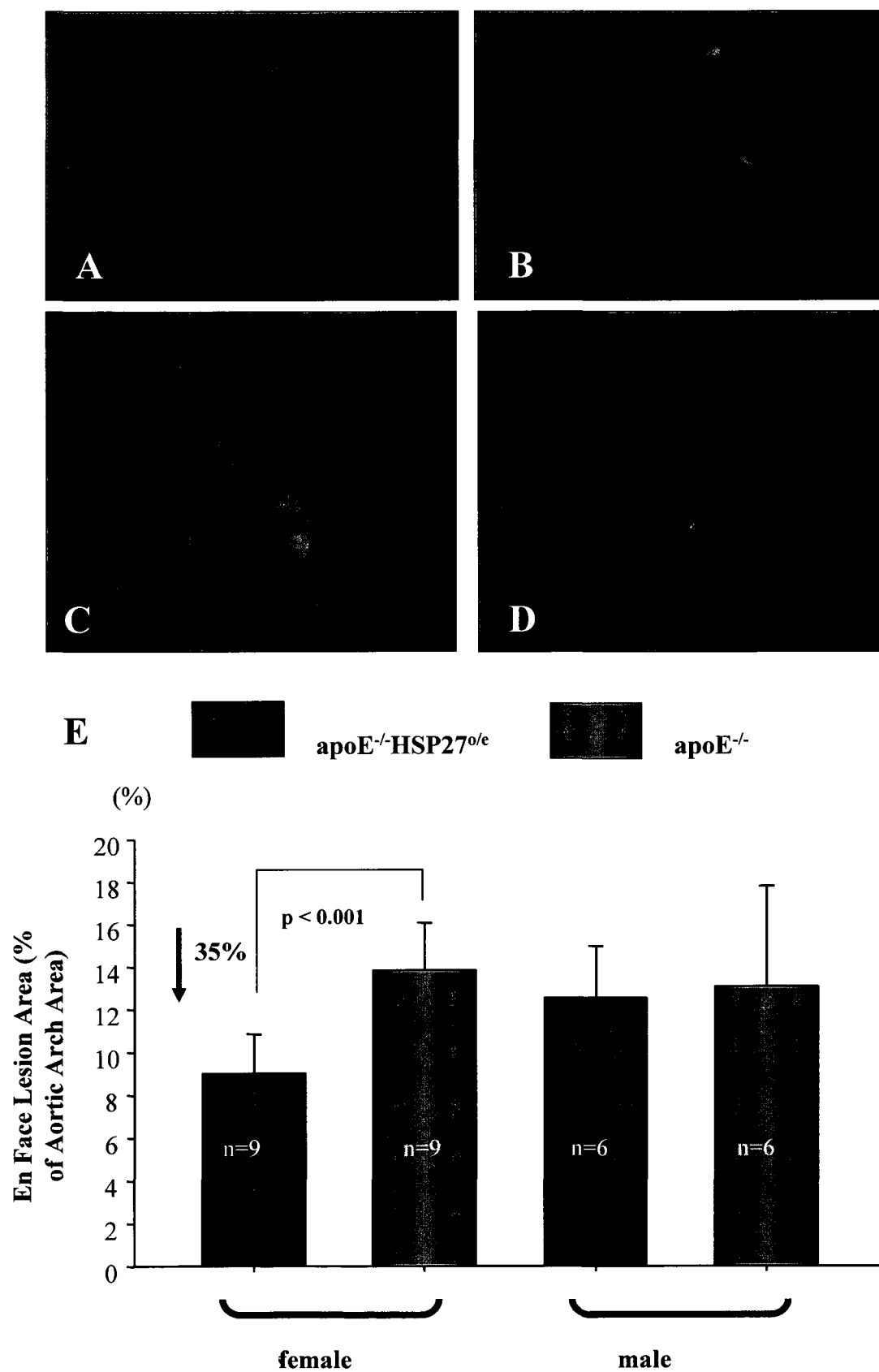
Using a mouse model of inflammatory atherosclerosis, we sought to determine if over-expression of human HSP27 (HSP27<sup>oe</sup>) is protective against atherogenesis in apoE<sup>-/-</sup> mice that are prone to atherosclerosis when fed a diet supplemented with cholesterol.(1)

HSP27<sup>oe</sup>apoE<sup>-/-</sup> and apoE<sup>-/-</sup> mice were placed on a high-cholesterol diet for 4 weeks and euthanized at 10-weeks of age. Mean body weight and length, as well as total serum cholesterol levels were similar for these two groups of mice (data not shown). The percentage aortic lesion area, measured by quantitative histomorphology of oil red O-stained *en face* specimens was 35% reduced in apoE<sup>-/-</sup>HSP27<sup>oe</sup> vs. apoE<sup>-/-</sup> female mice (n=9/group; p<0.001; **Figure 4.1**). The male apoE<sup>-/-</sup>HSP27<sup>oe</sup> and apoE<sup>-/-</sup> mice (n= 6/group) had similar percentage aortic lesion areas (12.9±1.2 % vs. 13.5± 1.0 % lesion area compared to total arch area, respectively; p=0.69). Serum HSP27 levels measured using an ELISA were low in all apoE<sup>-/-</sup>HSP27<sup>oe</sup> mice fed a normal chow diet (**Figure 4.2A**). However, following 4 weeks of a high-fat diet, female apoE<sup>-/-</sup>HSP27<sup>oe</sup> mice had more than 10-fold higher circulating levels of HSP27 compared to male mice (p≤0.05). This difference in serum HSP27 levels was not apparent until after 2 weeks of ingestion of a cholesterol-enriched diet (e.g. serum HSP27 levels after 2 weeks in females: 536±308 pg/ml vs. males: 123±81 pg/ml; p=0.456). When circulating HSP27 levels were compared to total *en face* lesion area, there was a remarkable inverse correlation between serum HSP27 levels and atherosclerotic lesion area (r<sup>2</sup>=0.78; p<0.001, **Figure 4.2B**) in both males and females. When only females were used for

**Figure 4.1: HSP27 over-expression reduces atherosclerotic lesion size in female mice.**

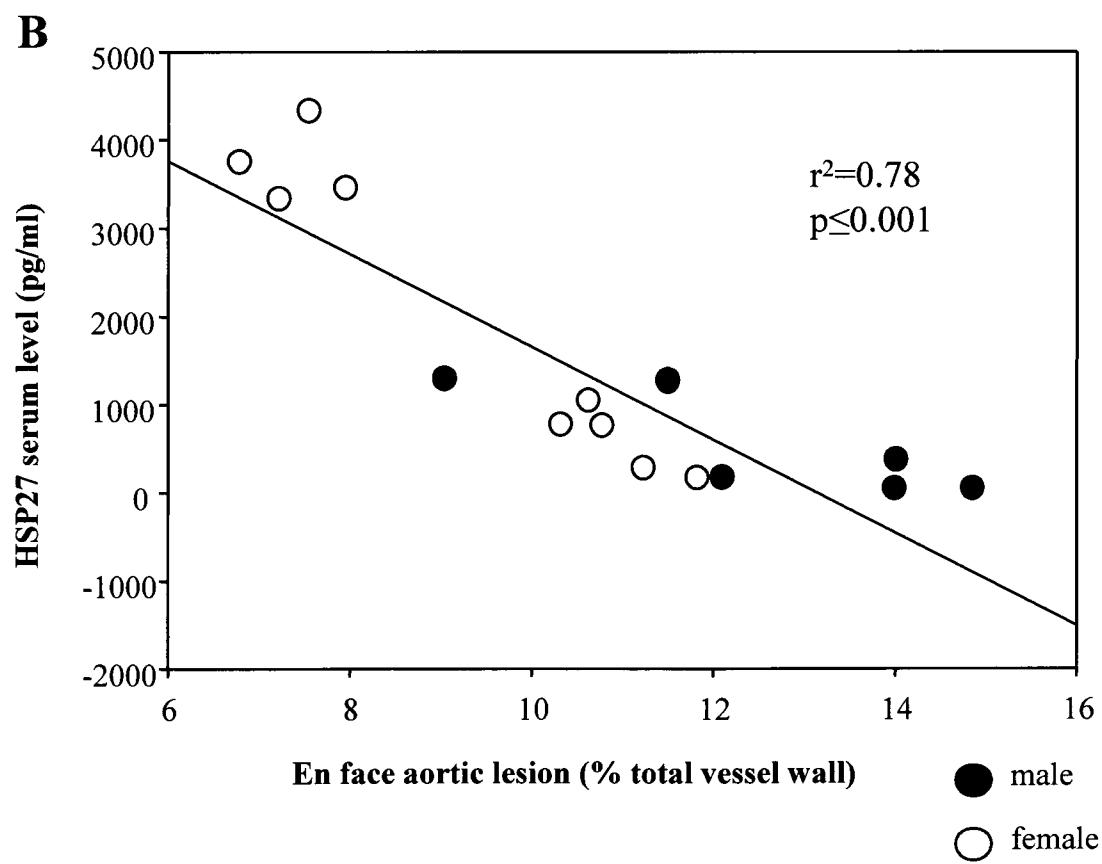
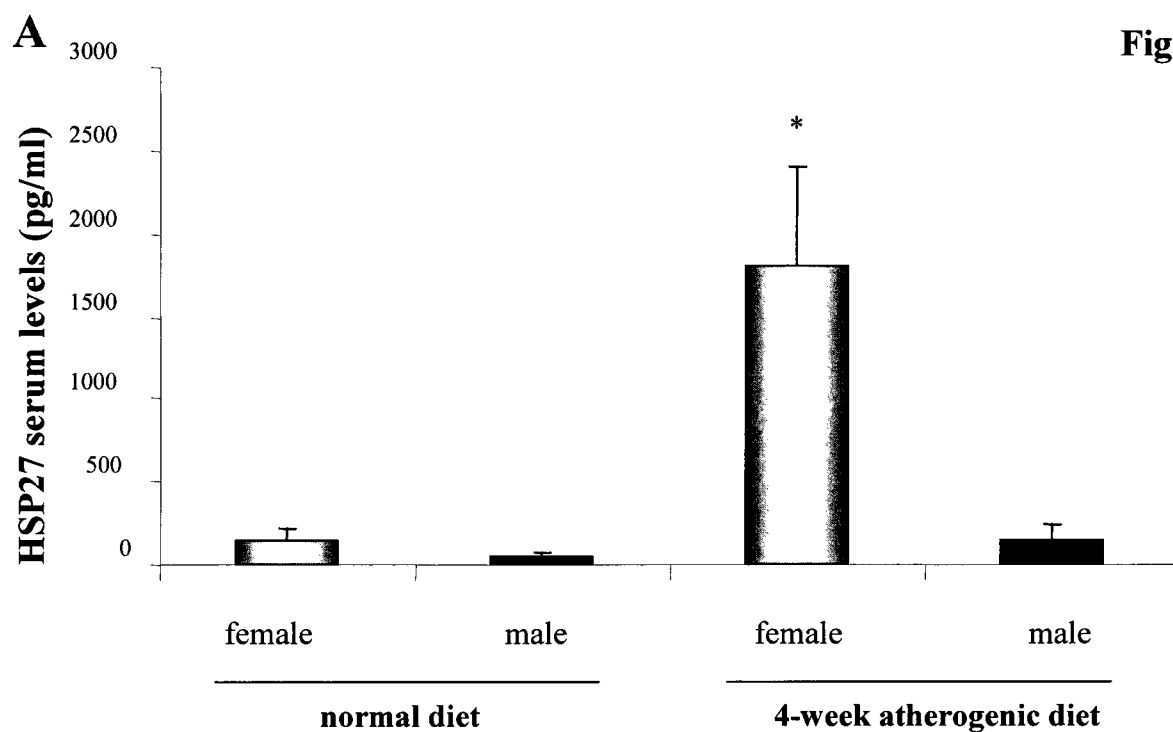
Total aortic en face atherosclerotic lesion area was analyzed in mice over-expressing human HSP27 (apoE<sup>-/-</sup>HSP27<sup>oe</sup>) (**A, C**) and compared to their apoE<sup>-/-</sup> littermates (**B, D**); pictured here are female mice. Quantification of lesion area/total aortic arch area (**E**) demonstrated a 35% reduction in lesion burden in HSP27<sup>oe</sup> apoE<sup>-/-</sup> compared to apoE<sup>-/-</sup> female mice (\*p<0.001). No difference was observed in the male mice.

**Figure 4.1**



**Figure 4.2:** (A) HSP27 serum levels increase in response to an atherogenic diet. Serum HSP27 levels from mice fed a normal chow diet (left columns) and from the same mice 4 weeks after ingesting a high-fat atherogenic diet (right columns) (\*  $p \leq 0.001$ ). (B) Regression analysis comparing HSP27 serum levels with aortic *en face* lesion area in male and female HSP27 over-expressing mice.

**Figure 4.2**



correlation analysis, the relationship between circulating HSP27 and atherosclerotic lesion area was even stronger ( $r^2=0.9$ ;  $p<0.001$ ).

### **HSP27 is Secreted *In Vitro* in Response to Estrogen and acLDL**

As serum HSP27 levels inversely correlate with aortic lesion area in mice fed a cholesterol-enriched diet, and is >10-fold higher in females than in males, we determined *in vitro* if HSP27 is released upon stimulation with estradiol (E2) and/or atherogenic acetylated low density lipoprotein (acLDL). Human macrophages (U937) were plated in replicates at a density of  $1 \times 10^6$  per well and treated with estradiol and/or acLDL. Conditioned medium was collected and prior to analysis for secreted HSP27, overall cell viability was measured using an LDH-release assay and revealed no difference in cell viability or membrane integrity between any of the treatments (data not shown). Treatment of macrophages with estrogen (E2) for 24 hours caused a dose-dependent increase in HSP27 release into the media compared to controls (**Figure 4.3A**)(13)(12). Estrogen-induced HSP27 release also increased over time, with maximum secretion after 24 hours (**Figure 4.3B**). Macrophages were subjected to increasing concentrations of acLDL (1 to 100  $\mu\text{g/ml}$ ) for 24 hours. HSP27 protein was detected by western blot in conditioned media from cells treated with 100  $\mu\text{g/ml}$  acLDL (**Figure 4.3C**). The addition of acLDL to the media containing estrogen caused a further increase in HSP27 secretion when compared to estrogen or acLDL treatment alone, indicating that these two mechanisms of secretion may act synergistically (**Figure 4.3D**). Upon treatment with estrogen, examination of intracellular protein levels revealed that HSP27 protein levels increased slightly in response to estrogen treatment, suggestive of an intracellular pool of HSP27 that is secreted without necessitating de novo protein synthesis (**Figure 4.3E**). Upon treatment with acLDL, there was a concomitant dose-dependent



**Figure 4.3: HSP27 is secreted from macrophages in response to estrogen and acLDL.**

(A). Human U937 macrophages in culture were treated with 10% charcoal-stripped FBS with or without increasing concentrations of 17 $\beta$ -estradiol (E2) for 24 hours, and conditioned media was subjected to western blotting using an antibody against HSP27. HSP27 secretion into the media increased in a dose-dependent manner with E2. \*  $p \leq 0.05$  compared to control.

(B). U937 cells were treated with or without 100nM E2 for 1, 3 and 24 hours, and conditioned media showed increasing HSP27 released into the extracellular space over time. \*  $p \leq 0.05$  compared to control; #  $p \leq 0.05$  compared to all treatment groups.

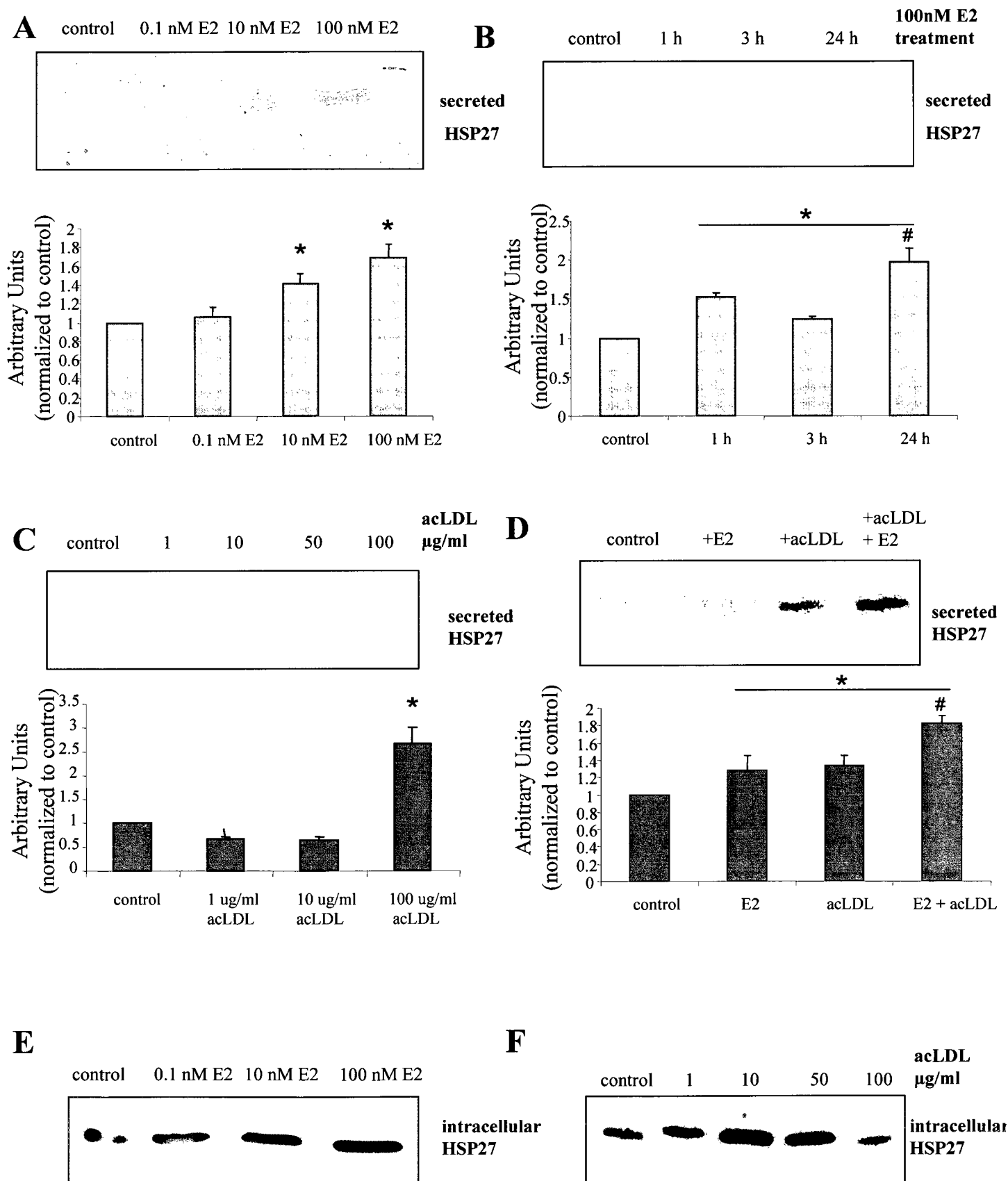
(C). U937 cells were treated in culture with increasing concentrations of acLDL (0-100  $\mu$ g/ml). HSP27 was detected in the conditioned media upon treatment with 100  $\mu$ g/ml acLDL (top panel). \*  $p \leq 0.05$  compared to control.

(D). U937 cells were treated with 100 nM E2 or 100  $\mu$ g/ml acLDL, or both together for 24 hours and conditioned media was analyzed by Western blot. HSP27 in the conditioned media increased with treatment of E2 and further increased with treatment of acLDL. An equal number of cells and volume of conditioned media was analyzed for each treatment condition for all conditioned media experiments. \*  $p \leq 0.05$  compared to control; #  $p \leq 0.05$  compared to all treatment groups.

(E) Intracellular protein from U937 cells treated with increasing concentrations of E2, and probed using an antibody to HSP27. Equal protein loading was confirmed using Ponceau S staining and  $\alpha$ -actin blotting.

(F) Intracellular protein from U937 cells treated with increasing concentrations of acLDL, and probed using an antibody to HSP27. Equal protein loading was confirmed using Ponceau S staining and  $\alpha$ -actin blotting.

**Figure 4.3**



increase in intracellular HSP27 expression, then an apparent decrease corresponding to increased HSP27 protein release into the media (**Figure 4.3F**).

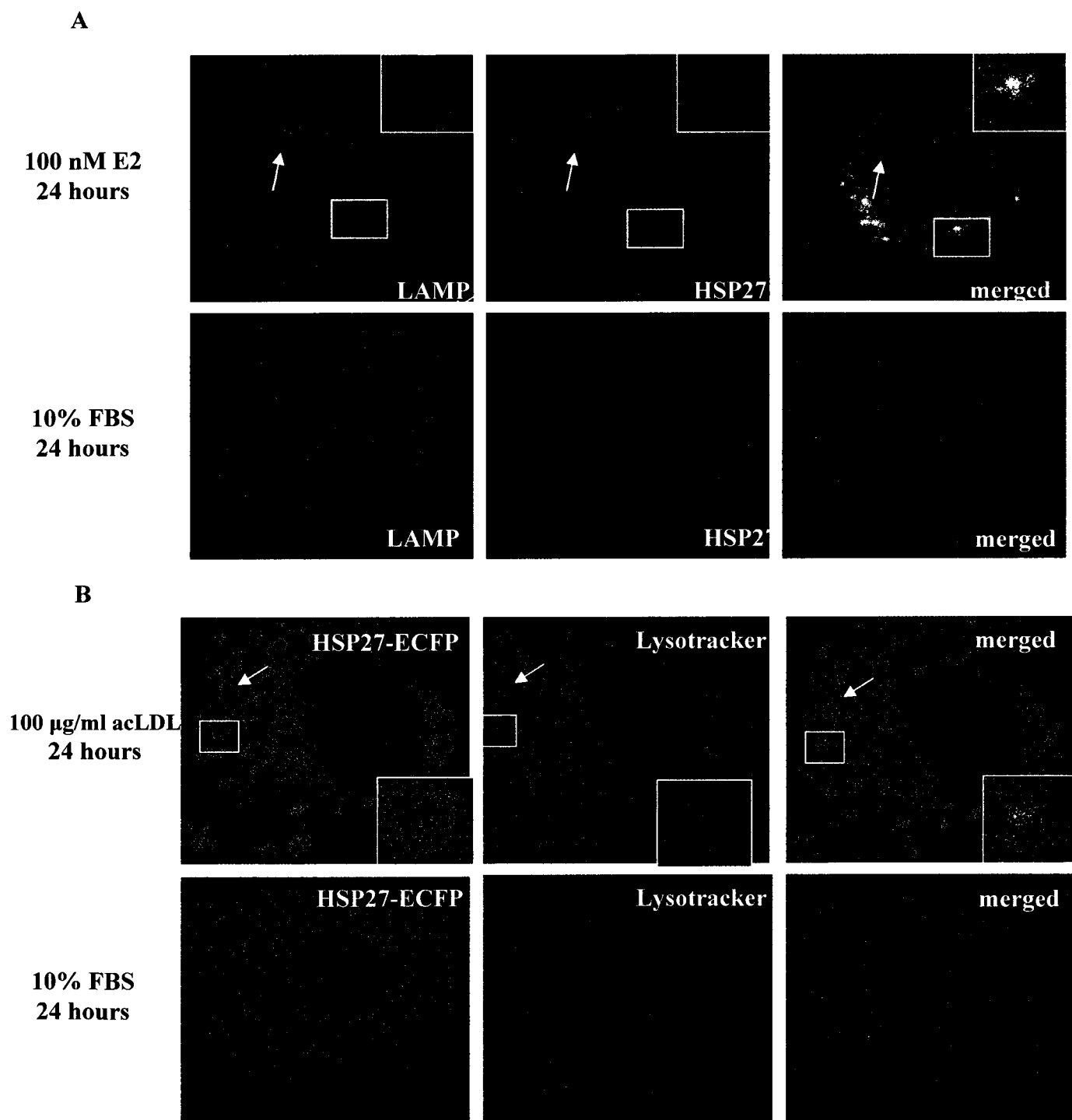
To examine the pathway of HSP27 secretion, we employed two independent experiments: the first used human U937 macrophages treated with E2 and immunolabeled for HSP27 and a marker of the lysosomal membrane (LAMP1); second, mouse J774 macrophages were transfected with a fluorescently tagged HSP27 (HSP27-ECFP), and treated with acLDL in the presence of LysoTracker, which labels acidic organelles (i.e. lysosomes) in live cells. Using both methods, we localized intracellular HSP27 in macrophage lysosomes after treatment with E2 or acLDL. Specifically, human macrophages treated with 100 nM E2 overnight displayed co-localization of HSP27 (red) and LAMP1 (green) (**Figure 4.4A**). Similarly, mouse macrophages transfected with fluorescently-tagged HSP27 (green) treated with 100 µg/ml acLDL and incubated in the presence of LysoTracker (red) displayed HSP27 co-localization within lysosomes after treatment for 1 hour and 24 hours (merged image, **Figure 4.4B**). Without estrogen treatment, HSP27 showed minimal co-localization with the lysosome (Figure 4A and 4B bottom row). Cells transfected with empty ECFP alone with or without acLDL treatment did not show co-localization with the lysosome, indicating that HSP27-ECFP was not simply degraded and targeted to the lysosome (data not shown). These results indicate that HSP27 is found within secretory lysosomal-like vesicles in macrophages under conditions which stimulate its secretion (e.g. upon treatment with E2 or acLDL).

#### **Extracellular HSP27 Binds the Scavenger Receptor-A and Prevents acLDL Uptake**

Given that HSP27 is secreted not only *in vitro* by atherogenic lipids, but *in vivo* in response to high fat diet, the next step was to determine if extracellular HSP27 is involved in

**Figure 4.4:** (A) U937 cells were treated with 100nM E2 overnight and immunolabeled using antibodies to LAMP1 (green, a marker for the lysosomal membrane) and HSP27 (red). Merged image (yellow) shows co-localization of HSP27 in the lysosome (magnification 100X). Images were obtained with sequential scanning to avoid bleed through, and arrows indicate locations which are positive for LAMP1 in the green channel and where no staining is observed in the red channel. (B). J774 cells transfected with HSP27-ECFP (green) were treated with 100 µg/ml acLDL 24 hours in the presence of LysoTracker red. Co-localization is seen as a yellow colour (magnification 100X). Images were obtained with sequential scanning to avoid bleed through, and arrows indicate locations which are positive for HSP27-ECFP in the green channel and where no staining is observed in the red channel. Experiments were performed in triplicate for all treatment conditions.

**Figure 4.4**



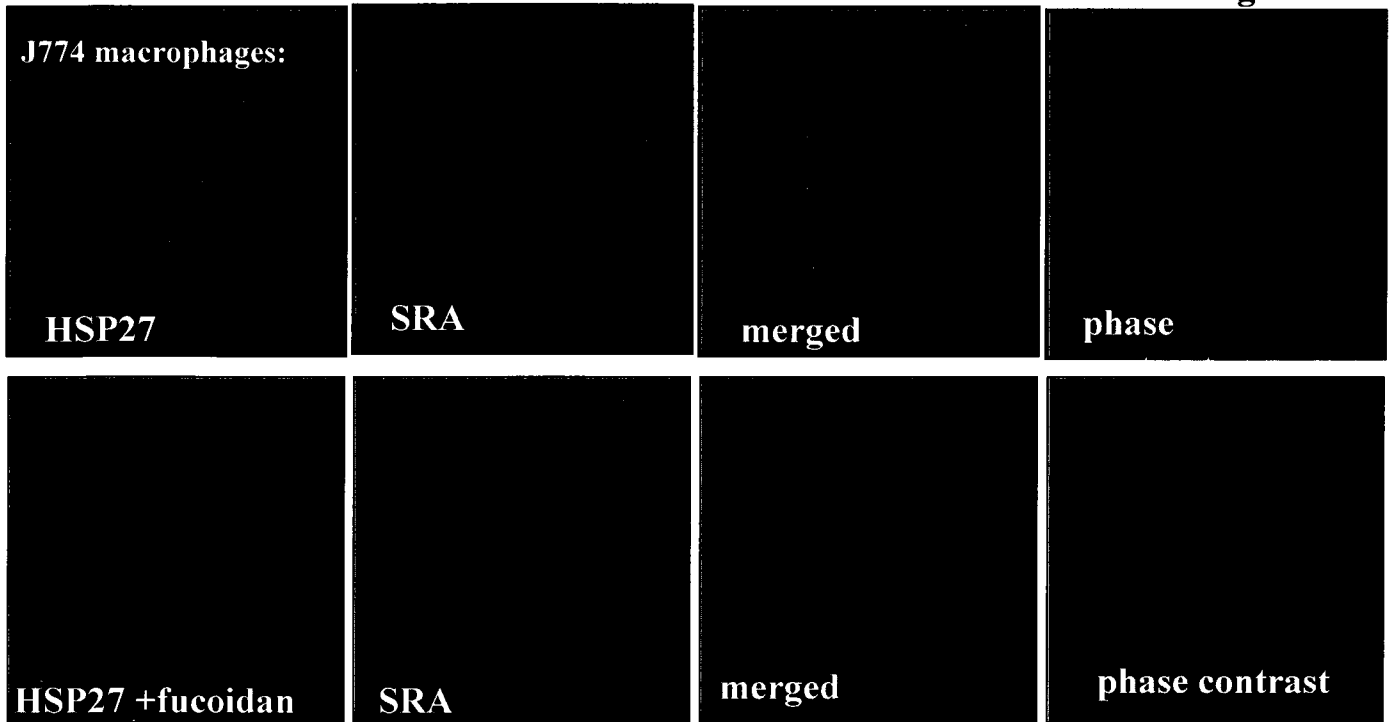
cholesterol trafficking and thus potentially the progression of atherosclerosis. Other groups have shown that members of the heat shock protein family (e.g. HSP70) bind a variety of cell surface receptors, including toll-like receptors and scavenger receptors (3,29-31). We investigated whether extracellular HSP27 binds the SR-A - an important receptor for the uptake of atherogenic lipids and the progression of atherosclerosis (16,23). Immunolabeling studies reveal that recombinant extracellular HSP27 is capable of binding the surface of macrophages, and co-localizes with SR-A (**Figure 4.5A**). In the presence of fucoidan, a specific competitor for SR-A, HSP27 binding to SR-A was reduced, indicating that this interaction is specific (11). Furthermore, in macrophages from SR-A<sup>-/-</sup> mice, HSP27 binding was absent (**Figure 4.5B**).

Next, we explored if endogenous HSP27 secreted in response to acLDL is capable of binding SR-A. Conditioned media from macrophages transfected with HSP27-ECFP (or empty ECFP) and treated with 100 µg/ml acLDL was applied to naïve, untreated macrophages at 4°C for 2 hours to allow HSP27-ECFP to bind the cell surface. Cells were then treated with DTSSP, a reversible, membrane-impermeable cross-linking agent, to cross-link HSP27 to the cell surface. Immunoprecipitation was carried out using antibodies to the fluorescent tag (anti-ECFP) and cross-linked proteins were reduced to reverse cross-linking, then separated on an SDS-PAGE gel and subjected to immunoblotting. Using antibodies to SR-A, we demonstrated that HSP27-ECFP is secreted from cells treated with acLDL and binds the SR-A under various concentrations of DTSSP (**Figure 4.5C**). Macrophage whole cell lysates were also probed and displayed a ~80 kDa band corresponding to SR-A in these cells.

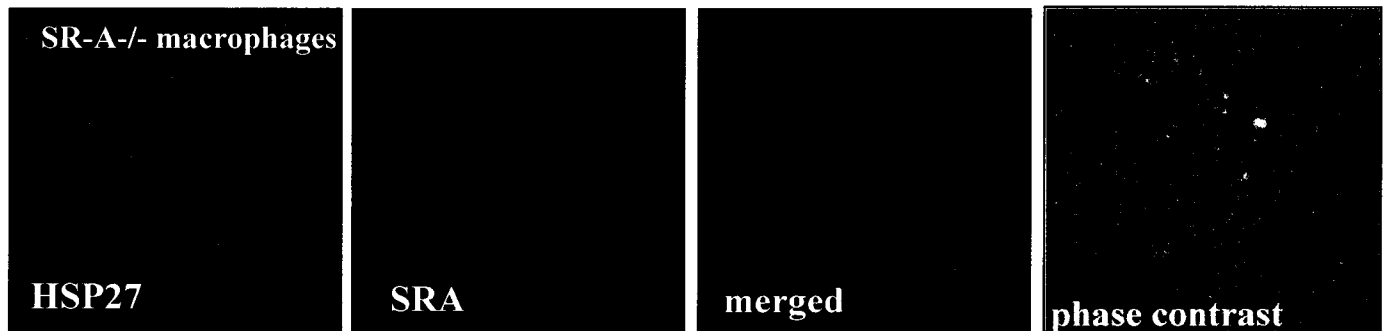
**Figure 4.5: Extracellular HSP27 co-localizes with the SR-A on the surface of macrophages.** (A). Recombinant HSP27 (5 µg/ml) was administered to J774 macrophages for 2 hours at 4°C. Immunolabeling of HSP27 (green) and SR-A (red) was visualized using confocal microscopy (100X). Cells were also treated with an SR-A specific competitive ligand (fucoidan, 10 µg/ml) before administration of HSP27 (bottom panel). Co-localization is seen as a yellow colour. (B). Macrophages from SR-A<sup>+/+</sup> (data not shown) and SR-A<sup>-/-</sup> mice were harvested and incubated with recombinant HSP27 (5 µg/ml) for 2 hours at 4°C. Immunolabeling was performed as described above. (C). HSP27-ECFP (or empty ECFP control; data not shown) secreted in response to acLDL was applied to naïve macrophages at 4°C for 2 hours. Cells were crosslinked with 0.2 mM and 2 mM DTSSP and immunoprecipitated using an antibody to ECFP. Lane containing non-immunoprecipitated cell lysate represents 25-30% of input (data not shown). Experiments were performed in triplicate for all treatment conditions.

**A**

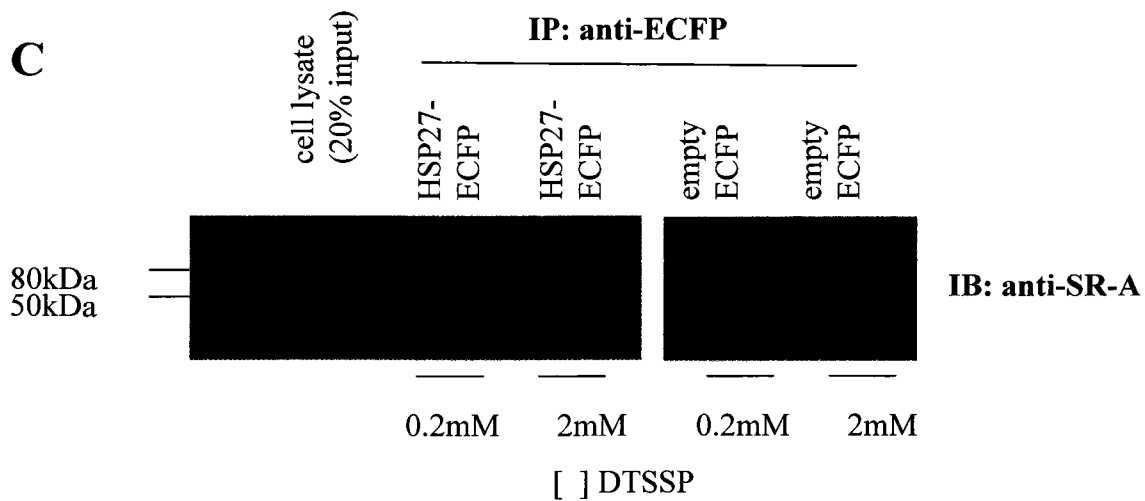
**Figure 4.5**



**B**



**C**



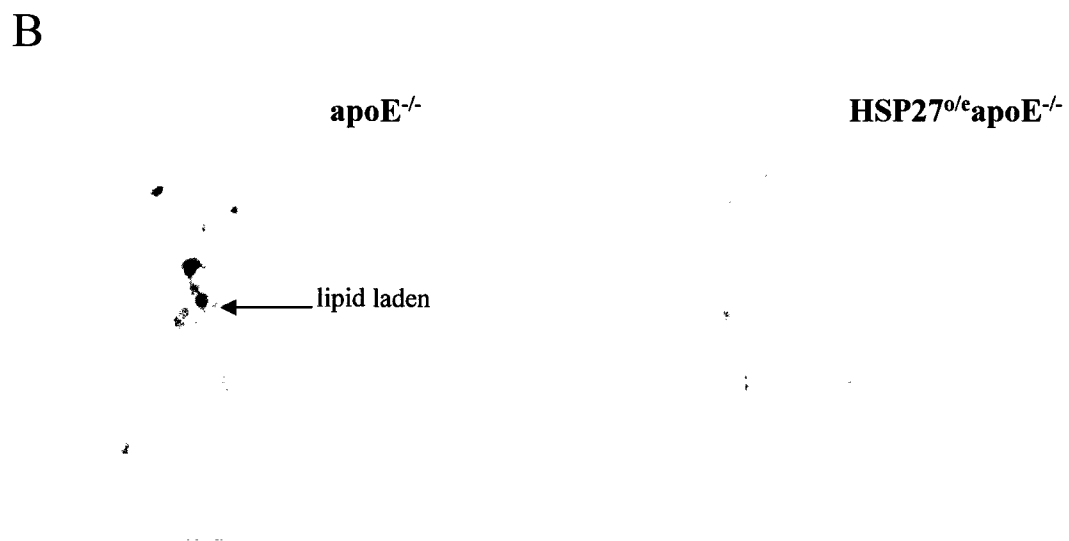
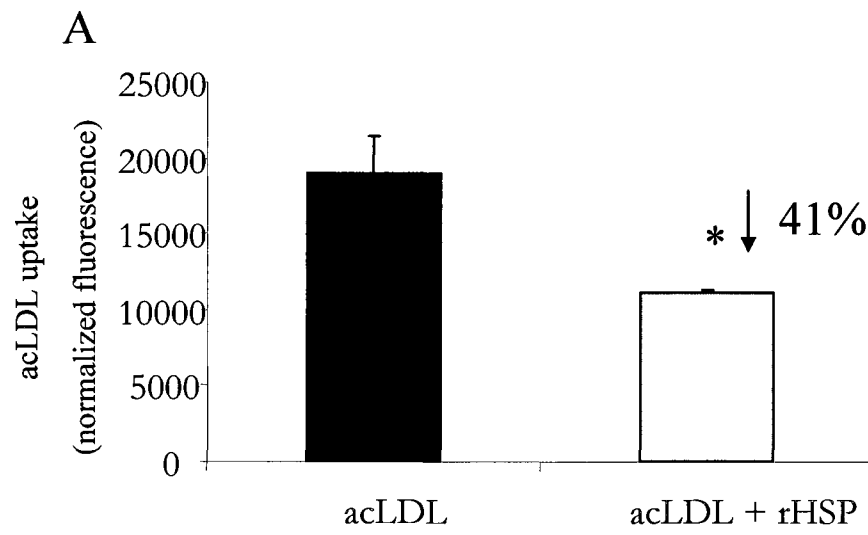


As foam cell formation is a hallmark of atherogenesis, and prevention of lipid uptake by macrophages may serve to reduce lesion development and vessel wall inflammation, we hypothesized that HSP27 binds the SR-A to prevent uptake of atherogenic lipids such as acLDL in order to attenuate foam cell formation. Mouse macrophages were cultured *in vitro* in the presence of fluorescently labeled acLDL for 6 hours, and acLDL uptake was measured using a fluorometer before being normalized for total cell number (n=10 wells per treatment; experiments performed in triplicate). When extracellular HSP27 was added to the culture media, there was a 41% reduction in specific acLDL uptake by macrophages ( $p \leq 0.05$ ; **Figure 4.6**; normalized to non-specific uptake in the presence of the SR-A competitor fucoidan). To investigate if a reduction in overall lipid uptake occurs *in vivo* in the presence of HSP27, peritoneal macrophages from HSP27<sup>o/e</sup>apoE<sup>-/-</sup> and apoE<sup>-/-</sup> mice were harvested and stained with Oil Red O, which stains intracellular lipids red. Semi-quantitative analysis was performed and the total number of lipid-laden cells were counted and expressed as a percentage of total cell number by two independent blind reviewers. Only 3% of macrophages from HSP27<sup>o/e</sup>apoE<sup>-/-</sup> were considered lipid-laden, whereas 21% of cells from apoE<sup>-/-</sup> mice were considered lipid-laden (**Figure 4.6B** and **Online Table 1**). Moreover, using western blotting we noted that the reduction in macrophage acLDL uptake by HSP27 attenuated acLDL-induced release of IL-1 $\beta$ , a potent pro-inflammatory cytokine (**Figure 4.7A**). Extracellular HSP27 also increased the release of the anti-inflammatory cytokine IL-10 (**Figure 4.7B**). Previous reports involving human monocytes suggest that HSP27 can induce the release of IL-10 but not TNF- $\alpha$ , and hence agree with our observations that extracellular HSP27 is primarily an anti-inflammatory signaling protein.(10) These results

**Figure 4.6: Extracellular HSP27 inhibits uptake of acLDL by macrophages via the SR-**

**A.** (A) Fluorescently labeled acLDL (5ug/ml) was added to macrophages in the presence (right column) or absence (left column) of recombinant HSP27 (5μg/ml). Cells were also treated with an SR-A specific competitor (fucoidan, 10ug/ml) before administration of acLDL or HSP27. Cells were harvested, and fluorescent acLDL uptake was measured using a fluorometer and normalized to cell number. Non-specific uptake in the presence of fucoidan was subtracted to define specific acLDL uptake; at least 10 samples were analyzed per group and comparisons were made using a One-Way ANOVA; \*  $p \leq 0.05$  compared to acLDL alone. (B) Peritoneal macrophages from apoE<sup>-/-</sup> and HSP27<sup>o/e</sup>apoE<sup>-/-</sup> mice were plated, fixed and stained for Oil Red O. Cells were counted and those that were “lipid-laden” (deep red colour) were expressed as a % of total cell number. HSP27<sup>o/e</sup>apoE<sup>-/-</sup> macrophages contained only 3% lipid-laden cells compared to apoE<sup>-/-</sup> macrophages which contained 21% lipid-laden cells (see **Table 4.1**).

**Figure 4.6**



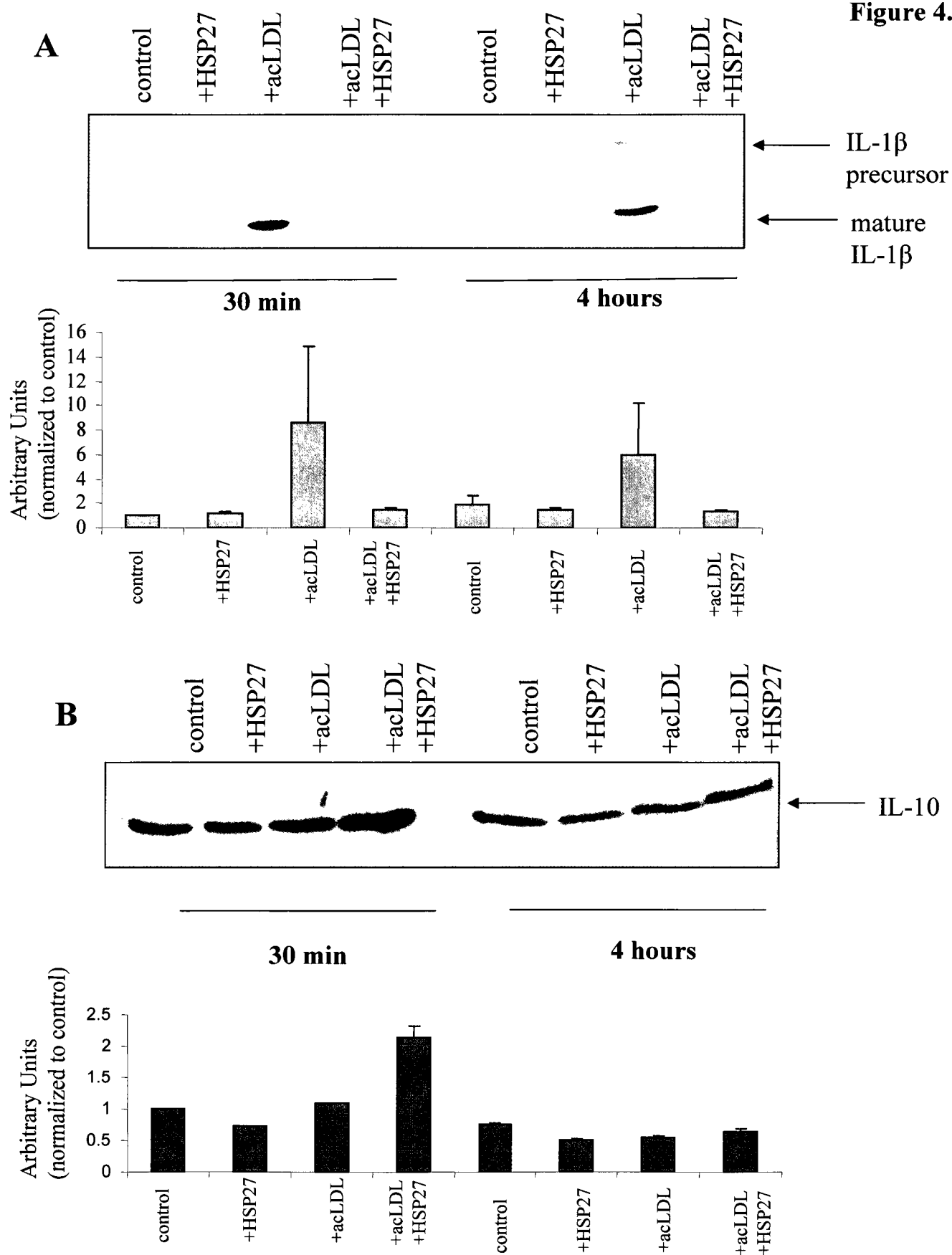
**Table 4.1:** Peritoneal macrophages from apoE<sup>-/-</sup> and HSP27<sup>0/e</sup>apoE<sup>-/-</sup> mice were plated, fixed and stained for Oil Red O. Cells were counted and those that were “lipid-laden” (deep red colour) were expressed as a % of total cell number. HSP27<sup>0/e</sup>apoE<sup>-/-</sup> macrophages contained only 3% lipid-laden cells compared to apoE<sup>-/-</sup> macrophages which contained 21% lipid-laden cells.

**Table 4.1**

| <b>apoE<sup>-/-</sup></b> |                |                   | <b>HSP27<sup>0/e</sup>apoE<sup>-/-</sup></b> |                   |
|---------------------------|----------------|-------------------|----------------------------------------------|-------------------|
|                           | <b># cells</b> | <b>% of total</b> | <b># cells</b>                               | <b>% of total</b> |
| normal                    | 90             | 78.9              | 158                                          | 96.9              |
| lipid laden               | 24             | <b>21.1</b>       | 5                                            | <b>3.1</b>        |
| total cell #              | 114            | 100               | 163                                          | 100               |

**Figure 4.7: Extracellular HSP27 results in release of cytokines involved in the inflammatory response.** Fluorescently labeled acLDL (5 ug/ml) was added to J774 macrophages in the presence or absence of recombinant HSP27 (5 µg/ml). Conditioned media was collected after 30 minutes and 4 hours and 40 µl was subjected to western blotting for IL-1β (A) and IL-10 (B). Addition of HSP27 caused a decrease in the acLDL-induced secretion of IL-1β into the extracellular space. An equal number of cells and volume of conditioned media was analyzed for each treatment condition for all conditioned media experiments. Experiments were performed in triplicate for all treatment conditions.

**Figure 4.7**



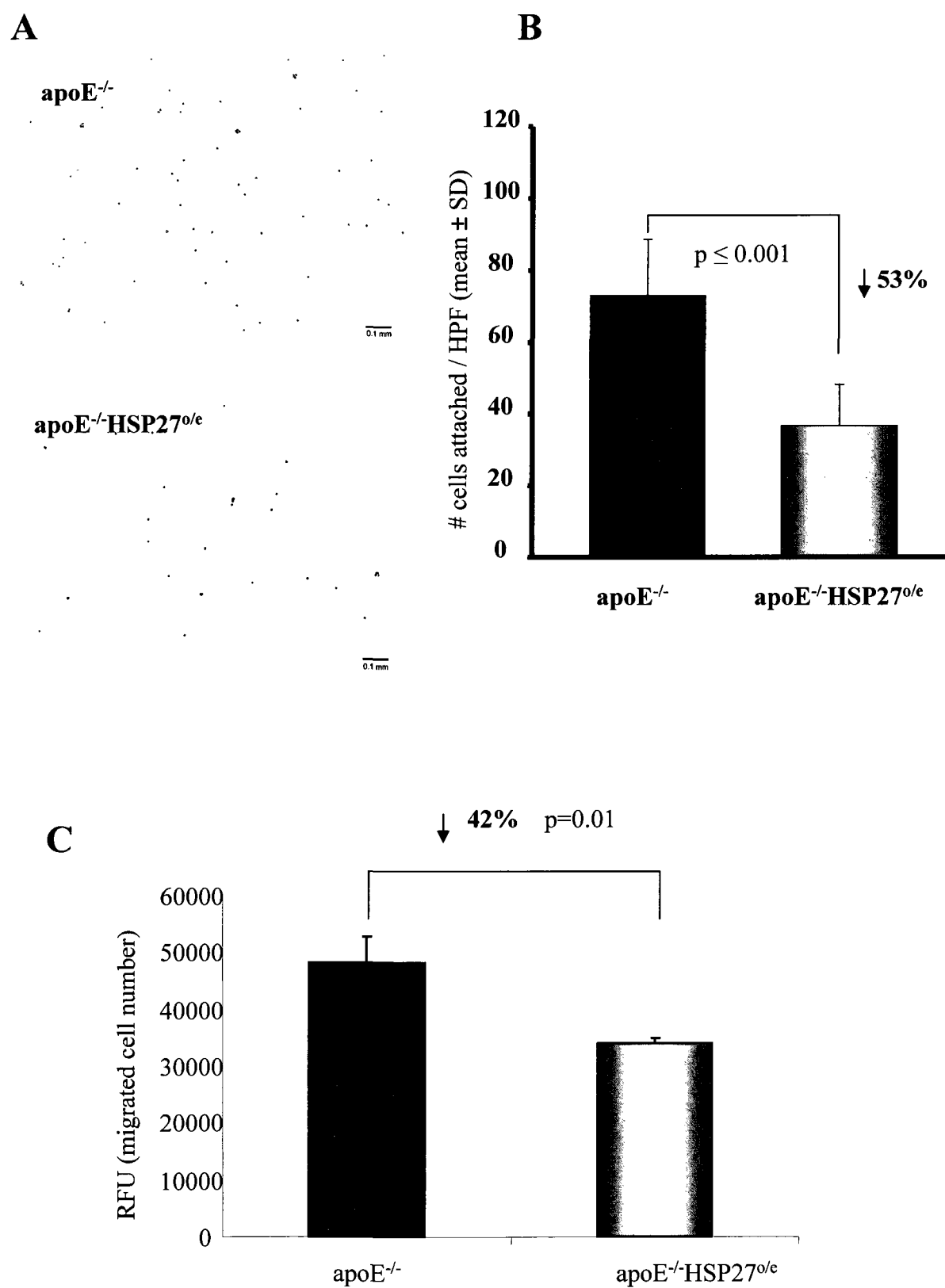
suggest that extracellular HSP27 is capable of reducing foam cell formation and the accompanying inflammation.

### **HSP27 Over-expression Reduces Cell Adhesion and Migration**

To further investigate how HSP27 might be protective against the development of atherosclerosis, peritoneal macrophages were harvested from apoE<sup>-/-</sup>HSP27<sup>o/e</sup> and apoE<sup>-/-</sup> mice after a high fat diet. Cells were plated in culture on a collagen matrix, and allowed to adhere for 2 hours. There was a 53% reduction in cell adhesion in apoE<sup>-/-</sup>HSP27<sup>o/e</sup> macrophages compared to apoE<sup>-/-</sup> (p≤0.001; **Figure 4.8A&B**). Macrophages were also maintained overnight in a transwell migration chamber and allowed to migrate towards a 10% FBS gradient. There was a 42% reduction in cell migration in apoE<sup>-/-</sup>HSP27<sup>o/e</sup> macrophages compared to apoE<sup>-/-</sup> (p≤0.01; **Figure 4.8C**). No cell migration was detectable in the absence of FBS (data not shown). Taken together, macrophages that over-express HSP27 display reduced adherence and migration *in vitro*, suggesting that *in vivo* these cells are less likely to incorporate into vascular lesions and exacerbate disease.



**Figure 4.8**



**Figure 4.8: HSP27 over-expression results in decreased macrophage adhesion and migration.** (A). Peritoneal macrophages harvested from apoE<sup>-/-</sup>HSP27<sup>o/e</sup> and apoE<sup>-/-</sup> mice were plated on type I collagen. After 2 hours incubation, the cells were washed and fixed. (B). Cell nuclei were stained with Hoechst 33258 and the number of cells per high power field (HPF) was manually counted. (C). Peritoneal macrophages as in (A) were subject to CytoSelect™ migration assay for 24 hours, and quantified as total number of cells migrated towards 10% FBS. Experiments were performed in triplicate for all treatment conditions.

## 4.7 Discussion

The present study is an extension of previous work by our group and others that demonstrate reduced tissue and serum levels of HSP27 with the development of atherosclerosis (21,22,24). However, in this manuscript we bring forth important new mechanistic information regarding the atheroprotective effects of HSP27. Specifically, we asked if over-expression of HSP27 is protective against the development of atherosclerotic lesions in apoE<sup>-/-</sup> mice fed a cholesterol enriched diet. Over-expression of HSP27 in apoE<sup>-/-</sup> mice resulted in a 35% decrease in aortic lesion area relative to apoE<sup>-/-</sup> mice – but only in females (p≤0.001). With the commencement of an atherosclerotic diet serum HSP27 levels remained low in male mice, yet showed a dramatic increase in females. Interestingly, there was a remarkable inverse correlation between serum HSP27 levels and lesion area in both male and female mice (r<sup>2</sup>=0.78; p<0.001); when females were examined independently of males, this relationship was even stronger (r<sup>2</sup>=0.90; p<0.001). We confirmed these *in vivo* observations by showing *in vitro* that both acLDL and estrogen cause the secretion of HSP27 into the extracellular space, where it binds the scavenger receptor A to prevent acLDL uptake and inflammatory foam cell formation.

A logical question arose from our initial observations on serum HSP27 levels in mice and humans: what is the mechanism by which HSP27 exits the cell? Given that HSP27 is primarily described as an intracellular protein and does not contain signal sequences that would sort it to a traditional secretory vesicle we reviewed our initial observations and hypothesized that perhaps estrogens combined with components of the atherogenic diet played a role in HSP27 release. While the promoter region of HSP27 does contain a

functional estrogen response element and that HSP27 expression is regulated by estrogen, (26,32) its secretion into the extracellular space is not known to be induced by ovarian hormones. Hence we approached the question of HSP27 release from the cell by examining the following three features. First, we looked at the structural means by which HSP27 might exit the cell, and found that, like HSP70 it was sorted in the cell into lysosome-like vesicles. Second, we demonstrated that estrogen, in both a dose- and time-dependent fashion, enhanced the secretion of HSP27 by macrophages. Finally, acLDL also promoted HSP27 release from the cell – and in an apparent synergistic manner with E2. Given that there was no change in membrane integrity or cell viability between treatments and controls (as measured by the LDH-release assay) we can conclude that these observed increases in HSP27 secretion into the media are not simply a result of cell necrosis or changes in membrane permeability, but rather by an active and potentially regulated secretion mechanism. Interestingly, cultured human smooth muscle cells did not secrete HSP27 in response to E2, while cultured human endothelial cells showed minimal release of HSP27 in response to E2 (data not shown).

Perhaps the most intriguing data from our studies is the discovery of the interaction between HSP27 and the SR-A. Not only is this interaction specific, as demonstrated using a competitor for SR-A as well as SR-A<sup>-/-</sup> macrophages, but HSP27 reduces the ability of SR-A on the surface of macrophages to engulf acLDL and acquire the foam cell phenotype. To our knowledge, this is the first evidence of a cell-surface receptor for HSP27. Previous reports demonstrate that some members of the heat shock protein family are capable of binding a variety of receptors, namely those involved in antigen recognition and immune signaling (reviewed in 5). For example, HSP70 can bind toll-like receptors 2 and 4 (TLR-2 and -4) and

thereby induce NF $\kappa$ B activation, IL-6 production, as well as the secretion of IL-1 $\beta$  and IL-12 - both pro-inflammatory cytokines (3,28). Other reports show that SR-A is capable of binding Gp96, an endoplasmic-reticulum bound HSP on antigen presenting cells (4). However, we now show that HSP27 may have effects opposite to those observed for other extracellular HSPs. When added to macrophages *in vitro*, HSP27 reduced acLDL-induced secretion of IL-1 $\beta$  and increased the secretion of IL-10; implying that HSP27 primarily results in anti-inflammatory cytokine induction. Indeed, apoE<sup>-/-</sup>HSP27<sup>o/e</sup> macrophages showed decreased cell adhesion and migration relative to peritoneal macrophages from apoE<sup>-/-</sup> mice. Taken together, these data suggest a unique atheroprotective mechanism by which extracellular HSP27 is capable of preventing the uptake of atherogenic lipids and reducing foam cell-induced inflammation.

While the available data appear to indicate that HSP27 is a novel biomarker of atherosclerosis and is atheroprotective, mechanistic insights remain elusive. From our previous studies we noted that HSP27 is an ER $\beta$  associated protein and, at least *in vitro*, is a co-repressor of estrogen mediated signaling (22,2). However, these studies purely focused on the intracellular role for HSP27 in estrogen transcriptional signaling. Currently, we are working towards understanding if ERs are involved in the regulation of HSP27 release from cells. Experiments involving ovariectomy and specific estrogen receptor modulation in HSP27<sup>o/e</sup>apoE<sup>-/-</sup> mice will help to resolve this question. Furthermore, it remains unclear if the levels of extracellular HSP27 are partly reflective of the intracellular levels of this protein, which may be released into the extracellular space. It is possible that an increase in intracellular HSP27 precedes an increase in extracellular HSP27, which can serve to prevent atherogenic lipid uptake and inflammation, resulting in protection from atherosclerosis

development. Hence, we hope to develop novel therapies to modulate HSP27 levels either intra- or extracellularly and/or attenuate levels of circulating anti-HSP27 autoantibodies) (35,25).

Naturally, certain limitations apply to our study. First, although we observed an increase in circulating levels of HSP27 in female mice following a high fat diet, we can only make conclusions about relative and not absolute levels of HSP27. As seen in patients with normal coronary arteries, there are relatively high levels of HSP27 in healthy males and females (9,21). It is possible that in healthy mice, their levels of HSP27 are below the sensitivity of our assay and therefore reported as undetectable. Regardless, we still note a relative increase in serum HSP27 levels in female mice on a high-cholesterol diet compared to males and/or mice on a normal diet. Second, although we observe the co-localization of HSP27 within lysosomal-like vesicles following treatment with acLDL and estrogen, the specific participation of the lysosome in this pathway is still unknown. The exact cellular mechanisms by which HSP27 exits the cell certainly warrants further study.

In summary, we propose a novel mechanism of HSP27 atheroprotection that involves estrogen-induced attenuation of inflammation and inhibition of cholesterol uptake via the interaction of HSP27 with SR-A. While the atheroprotective effects of estrogens are well-recognized in animal models, our data suggest that estrogen-induced *release* of HSP27 may in fact be an important mechanism by which estrogens exert their beneficial effects on atherogenesis (14). Clearly the implications of this unique role for HSP27 are far-reaching, and highlight the possibility that HSP27 may be a new target for therapeutic modulation. While intracellular HSP27 protein levels are known to be induced by estrogen,(26) there may

be important subtleties with regards to how selective estrogen receptor modulation may trigger the release of atheroprotective HSP27; hence this is an area of ongoing research.

#### **4.8 Acknowledgements:**

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## **5.0 MANUSCRIPT # 4:**

### **Heat Shock Protein 27 Protects Against Atherogenesis via an Estrogen-Dependent Mechanism: Role of Estrogen Receptor Beta**

Authors: Katey Rayner, Jinagfeng Sun, Yong-Xiang Chen, Melissa McNulty, Trevor Simard, Xioaling Zhao, Dominic J. Wells, Jacqueline de Belleruche, Edward R. O'Brien.

*Arteriosclerosis, Thrombosis and Vascular Biology*

Submitted as a Brief Report: February 3<sup>rd</sup>, 2009.

#### **5.1 Significance of this Manuscript**

This manuscript directly answers the questions raised by our initial studies showing HSP27 protection from atherosclerosis is estrogen-dependent. Specifically, we show that after ovariectomy in the absence of endogenous estrogen, HSP27 protection from atherosclerosis is abolished. Similarly, replacement of estrogen recapitulates the 35% reduction previously observed in intact female mice. We also show both *in vitro* and *in vivo* that ER $\beta$  is the receptor primarily responsible for the release of HSP27 into the extracellular space. These results confirm our previous observations that HSP27 is indeed released into the serum in response to estrogen, and when levels in the serum are high, there is a protection from atherosclerosis. This study also uncovers a role for ER $\beta$  in estrogen-mediated atheroprotection.

#### **5.2 Author contributions:**

As first author of this manuscript, I conducted the surgical procedures, aortic dissection, lesion analysis and quantification. However, I was trained immensely by Jianfeng Sun and Yong-Xiang Chen. They also assisted with the analysis of the data. The other co-authors contributed both technical assistance and critical evaluation of the manuscript. The design of the experiments and writing of the manuscript was done in conjunction with Dr. O'Brien.

### 5.3 Abstract

**Objective:** We recently noted that over-expression of heat shock protein 27 (HSP27) is atheroprotective likely via novel extracellular pathways that involve the interaction with scavenger receptor-A. Interestingly, these effects were only observed in female mice, where there was an inverse relationship between serum HSP27 levels and aortic lesion burden. In the current study we sought to address whether estrogen is required for the observed atheroprotective benefits of HSP27 and for its release into the extracellular space. **Methods and Results:** After ovariectomy, HSP27 over-expression no longer resulted in elevated serum HSP27 levels and aortic atheroprotection in apoE<sup>-/-</sup> mice fed a high fat diet for 4 weeks. When the same mice were administered 17 $\beta$ -estradiol or the ER $\beta$ -specific agonist DPN aortic lesion area was reduced by 35% and 28% in apoE<sup>-/-</sup> mice that over-expressed HSP27 compared to non-over-expressing littermates, and was accompanied by increases in serum HSP27 levels of >4-fold and >3-fold; respectively. **Conclusions:** HSP27-mediated atheroprotection and release into the serum is estrogen-dependent and primarily mediated via ER $\beta$ . These results suggest that HSP27 may provide a novel therapeutic target for estrogen receptor specific modulation for the treatment and prevention of cardiovascular disease.

**Keywords:** heat shock proteins, estrogen, atherosclerosis, ER $\beta$

We initially discovered Heat shock protein 27 (HSP27) to be an estrogen receptor beta (ER $\beta$ ) associated protein (1,6) and more recently an independent atheroprotective protein found in the serum that interacts with Scavenger Receptor-A (SR-A) (7). Over-expression of HSP27 in apoE<sup>-/-</sup> mice fed a high fat diet (HFD) for 4 weeks leads to reduced aortic atherosclerotic plaque area and an increase in serum HSP27 levels that inversely correlate with lesion area – but only in female mice (7). Hence, we now seek to determine if estrogens are required for the atheroprotective effects of HSP27.



## **5.4 Methods**

### **Chemicals and Reagents**

All chemicals and reagents were purchased from Sigma unless otherwise noted.

Diarylpropionitrile (DPN) and propyl pyrazole triol (PPT) and ICI 182,780 were obtained from Tocris Bioscience (Ellisville, Missouri).

### **Ovariectomy model**

All animals used in this study were approved by the University of Ottawa Animal Care and Veterinary Service Committee. For all surgical procedures, mice were placed under a 3% isoflurane/oxygen mixture and buprenorphine was administered twice daily for three days post-surgery for analgesia. Female mice 6 weeks of age were incised dorsally and ovaries removed bilaterally. Mice were allowed to recover for 1 week, at which point pellets containing either 17 $\beta$ -estradiol (0.25 mg, 60-day release) or diarylpropionitrile (DPN; 0.25 mg, 60-day release) were implanted subcutaneously (Innovative Research America; Sarasota, FL) using a trochar (7-9 mice/group).

### **Mouse Atherosclerosis Model**

Mice over-expressing HSP27 (HSP27<sup>o/e</sup>) were generated at Imperial College London as previously described (1,3). HSP27<sup>o/e</sup> mice were then crossed more than 8 generations onto an apoE<sup>-/-</sup> C57BL6 background to generate HSP27<sup>o/e</sup>apoE<sup>-/-</sup> and apoE<sup>-/-</sup> littermates.

Genotyping was done in all animals for verification of both HSP27 and apoE<sup>-/-</sup> using PCR as previously described (2,3). Over-expression of HSP27 was verified using immunocytochemistry (using an antibody that recognizes both HSP25/HSP27), western

blotting (using an antibody that recognizes the HA-tag on the HSP27 protein) and real-time PCR (using a UPL probe and primers specific for HSP27). One week post pellet implantation (at 8 weeks of age), animals were placed on a high-fat diet containing 1.25% cholesterol and 15.8% fat (Harlan Teklad, Madison, WI) for 4 weeks. At euthanasia, animals were anaesthetized under isoflurane, and whole blood was collected through cardiac puncture. Hearts were perfused with phosphate-buffered saline (PBS) followed by 10% neutral buffered formalin (NBF) via the left ventricle, and the heart and aorta were removed and immersed in NBF overnight. Adventitial tissue was removed, and the aorta was opened longitudinally and stained with oil red O. Images were captured with a video camera and en face atherosclerotic lesions were analyzed by two observers using Image-Pro software (Media Cybernetics, Silver Spring, MD) and expressed as a percent lesion area per total area of the aortic arch. Uteri were removed and dissected of all fat and connective tissue before wet weight was obtained and expressed as a fraction of total body weight at sacrifice (see **Table 5-I & II**). Serum was collected and analyzed for total cholesterol (Wako Pure Chemical Industries, Ltd, Osaka, Japan) (see **Table 5-III**).

### **HSP27 Serum Levels**

Plasma levels of HSP27 were measured using an ELISA kit specific to human HSP27 (QIA119, Calbiochem, San Diego, CA). A total of 5  $\mu$ l of serum from HSP27<sup>oe</sup>apoE<sup>-/-</sup> and apoE<sup>-/-</sup> mice was diluted 1:10 in dilution buffer, and assayed according to the manufacturers' protocol. A standard curve of known amounts of HSP27 was constructed with each assay. This assay was found to have no cross-reactivity with mouse HSP25.

**Table I:** Body weight (g) at time of sacrifice (mean  $\pm$  SEM)

| Group    | apoE <sup>-/-</sup> | HSP27 <sup>o/e</sup> apoE <sup>-/-</sup> |
|----------|---------------------|------------------------------------------|
| OVX      | 24.4 $\pm$ 0.5      | 22.3 $\pm$ 0.9                           |
| OVX +E2  | 25.1 $\pm$ 0.5      | 23.7 $\pm$ 0.5                           |
| OVX +DPN | 26.1 $\pm$ 0.8      | 25.0 $\pm$ 0.4                           |

**Table II:** Uterine weight normalized to body weight (mg/g) at sacrifice (mean  $\pm$  SEM)

| Group    | apoE <sup>-/-</sup> | HSP27 <sup>o/e</sup> apoE <sup>-/-</sup> |
|----------|---------------------|------------------------------------------|
| OVX      | 0.75 $\pm$ 0.1      | 0.53 $\pm$ 0.02                          |
| OVX +E2  | 12.1 $\pm$ 0.6*     | 10.4 $\pm$ 0.6*                          |
| OVX +DPN | 0.6 $\pm$ 0.06      | 0.7 $\pm$ 0.09                           |

\* p $\leq$ 0.05 vs. all groups

**Table III:** Total serum cholesterol (mg/dL) at time of sacrifice (mean  $\pm$  SEM)

| Group    | apoE <sup>-/-</sup> | HSP27 <sup>o/e</sup> apoE <sup>-/-</sup> |
|----------|---------------------|------------------------------------------|
| OVX      | 1599.2 $\pm$ 118.2  | 1483.3 $\pm$ 91.5                        |
| OVX +E2  | 876.5 $\pm$ 82.8*   | 1034.2 $\pm$ 95.7*                       |
| OVX +DPN | 1266.7 $\pm$ 161.8  | 1558.8 $\pm$ 119.5                       |

\* p $\leq$ 0.05 vs. all groups

## **Cell Culture**

THP-1 human macrophages (American Type Culture Collection, ATCC; Manassas, Va) were cultured in RPMI 1640 (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, Wisent, Saint-Jean-Baptiste de Rouville, QC), sodium pyruvate, glutamine, penicillin-streptomycin fungizone,  $\beta$ -mercaptoethanol (Invitrogen).

Differentiation was induced with 100nM phorbol myristate acetate (PMA) for 72 hours before any treatment was initiated. Twenty-four hours before treatment, cells were placed in phenol-red free media containing 10% charcoal-stripped FBS. Cells were treated with E2 (100 nM), ICI (10 nM), PPT (10 nM) or DPN (10 nM) overnight unless otherwise stated. Conditioned media were analyzed for HSP27 secretion or cytokine expression after verification of cell viability using the LDH release assay (CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay; Promega, San Luis Obispo, CA) and equal cell number was plated per well. LDH release (a measure of cell injury) did not differ between any of the treatments, and was not significantly different than cell culture media alone (without cells).

## **Western Blotting**

For western blotting, 40  $\mu$ l of conditioned media were loaded onto a 10% SDS-PAGE gel and separated at 120 V using gel electrophoresis. Protein was then transferred to a PVDF membrane (BioRad, Hercules, CA) for 2 hours at 60 V. Membranes were then subjected to western blotting using monoclonal antibodies to HSP27 (Chemicon, 1:200).

## **Statistical Analysis**

All data represent mean  $\pm$  SEM, except as specifically stated. Each experiment was conducted at least 3 times. Statistical analysis was performed with one-way ANOVA by using SigmaStat 3.5 software. Differences were considered significant at p-value  $<0.05$  and is denoted by an asterisk (\*).

## 5.5 Results

### **HSP27 is Released into the Extracellular Space via ER $\beta$**

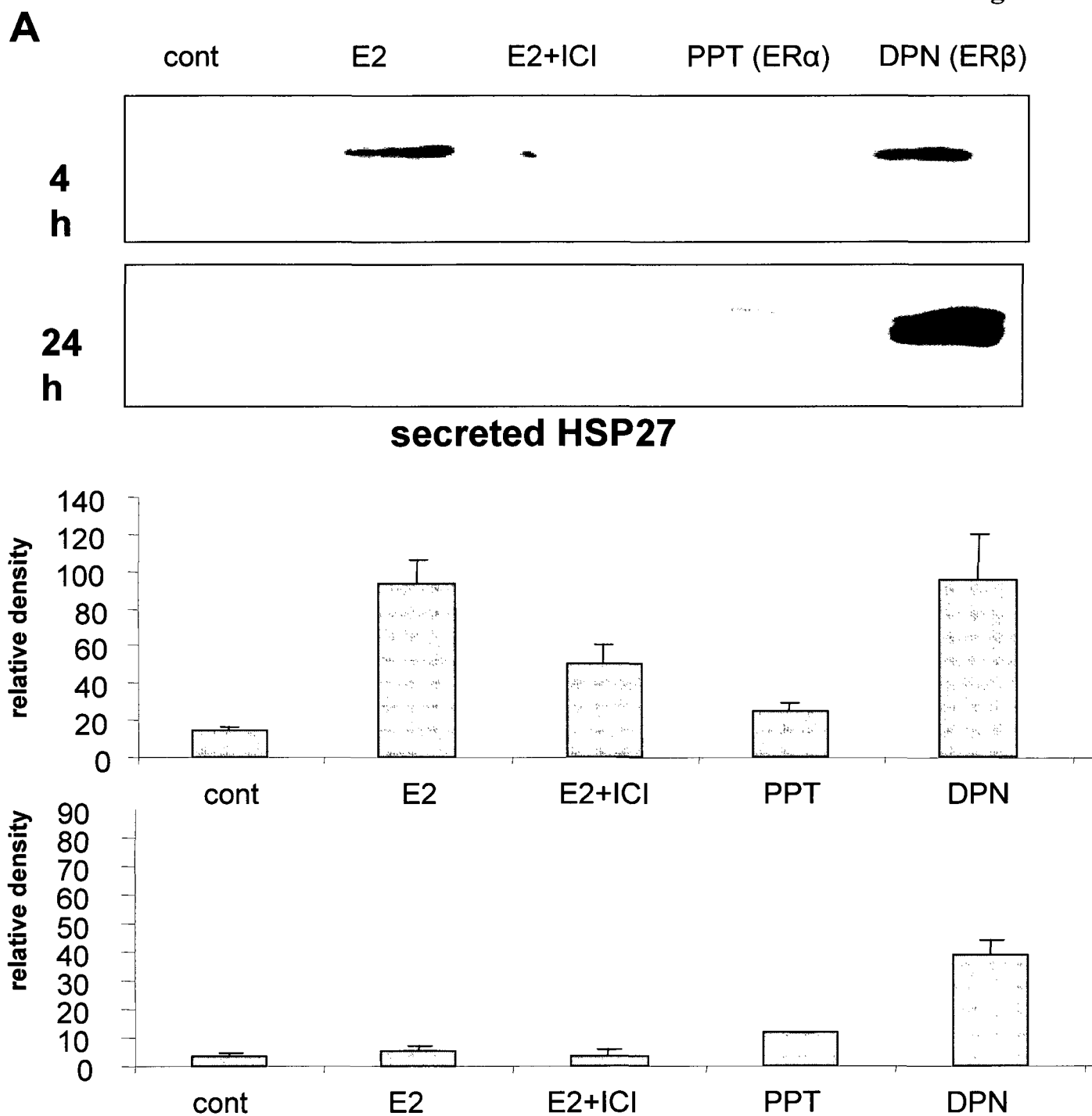
Given our previous observations that HSP27 in the extracellular space is atheroprotective and its release from cells appears to be induced via estrogen, we sought to determine which estrogen receptor (i.e. ER $\alpha$  or ER $\beta$ ) is responsible for the extracellular release of HSP27. Human macrophages were treated in culture with 17 $\beta$ -estradiol (E2; 1-100 nM) in the presence or absence of the ER antagonist ICI 182,780 (ICI). Treatment with E2 caused a dose-dependent release of HSP27 into the conditioned media that was almost completely abolished upon treatment with ICI (**Figure 5.1**). Macrophages were then treated for 4 hours with ER $\alpha$  or ER $\beta$  specific modulators (10 nM PPT and 10 nM DPN, respectively). While PPT resulted in little change compared to control conditions, DPN was approximately equivalent to E2 in effecting the release of HSP27 from macrophages -with these results being even more pronounced after 24 hours of treatment (**Figure 5.1**). Lactose dehydrogenase (LDH) levels in the conditioned media were similar in all groups (data not shown), thereby suggesting that differences in HSP27 release were unrelated to cell death.

### **Estrogen is required *in vivo* for HSP27-mediated atheroprotection**

Previously we demonstrated that female (but not male) mice over-expressing HSP27 had 35% smaller atherosclerotic lesions compared to non-overexpressing mice.(7) We therefore asked if estrogens are required for this observed effect by subjecting 6-week old female apoE<sup>-/-</sup> and HSP27<sup>oe</sup>apoE<sup>-/-</sup> mice to bilateral ovariectomy, followed by a 4 week high-fat diet. The *en face* aortic arch was stained with oil red O in order to permit quantification of

**Figure 5.1: Estrogen receptor  $\beta$  is primarily responsible for HSP27 secretion.** Human macrophages in culture were treated with 17 $\beta$ -estradiol (E2), E2 plus the pan estrogen receptor antagonist ICI 182,780 (E2+ICI), the ER $\alpha$ -specific agonist PPT or the ER $\beta$ -specific agonist DPN for 4 or 24 hours and secreted HSP27 was detected using western blotting.

**Figure 5.1**





the lipid-laden atherosclerotic area. In the absence of estrogen, HSP27<sup>o/e</sup>apoE<sup>-/-</sup> mice no longer enjoyed relative protection against lesion development and had a plaque burden that was similar to apoE<sup>-/-</sup> mice (e.g.,  $11.64 \pm 0.9\%$  vs.  $11.66 \pm 1.3\%$  of total arch area;  $p=0.99$ ;  $n=8$  mice/group; **Figure 5.2A & B**). To examine if this atheroprotection could be restored with replacement of estrogen, both groups of mice were implanted with a subcutaneous time-release E2 pellet (0.25 mg; approximately 4.2 $\mu$ g of estrogen released per day) one week prior to commencing a HFD. ApoE<sup>-/-</sup> mice supplemented with E2 showed reduced lesion size ( $3.70 \pm 0.35\%$  of total arch area,  $p \leq 0.05$ ) compared to the non-treated mice – consistent with previous reports (2,5). However, relative to the apoE<sup>-/-</sup> mice, E2 replacement therapy in the HSP27<sup>o/e</sup>apoE<sup>-/-</sup> mice resulted in an additional 34% reduction in lesion size ( $2.47 \pm 0.4\%$  of total arch area;  $p \leq 0.05$ ).

Given that HSP27 release into the extracellular space occurs upon stimulation with DPN (an ER $\beta$ -specific agonist) but not PPT (an ER $\alpha$ -specific agonist), we next asked whether DPN could affect the HSP27-mediated atheroprotection in our ovariectomized mouse model. DPN pellets (0.25mg) were implanted subcutaneously in an identical fashion to the E2 pellets. After 4 weeks of a HFD the lesions from HSP27<sup>o/e</sup>apoE<sup>-/-</sup> mice treated with DPN were 28% smaller than DPN-treated apoE<sup>-/-</sup> mice ( $8.8 \pm 0.5\%$  vs.  $12.2 \pm 0.8\%$ ,  $p \leq 0.05$ ).

### **HSP27 Release Into the Extracellular Space is Dependent Upon Estrogen**

To determine if estrogen is required for the release of HSP27 into the extracellular space *in vivo*, serum HSP27 levels were measured in all HSP27<sup>o/e</sup>apoE<sup>-/-</sup> mice at baseline, post-ovariectomy (i.e. prior to hormone supplementation), as well as 2 and 4 weeks following ingestion of a HFD. Following ovariectomy and throughout the course of the 4-week

**Figure 5.2: HSP27-Mediated Protection from Atherosclerosis Requires Estrogen.**

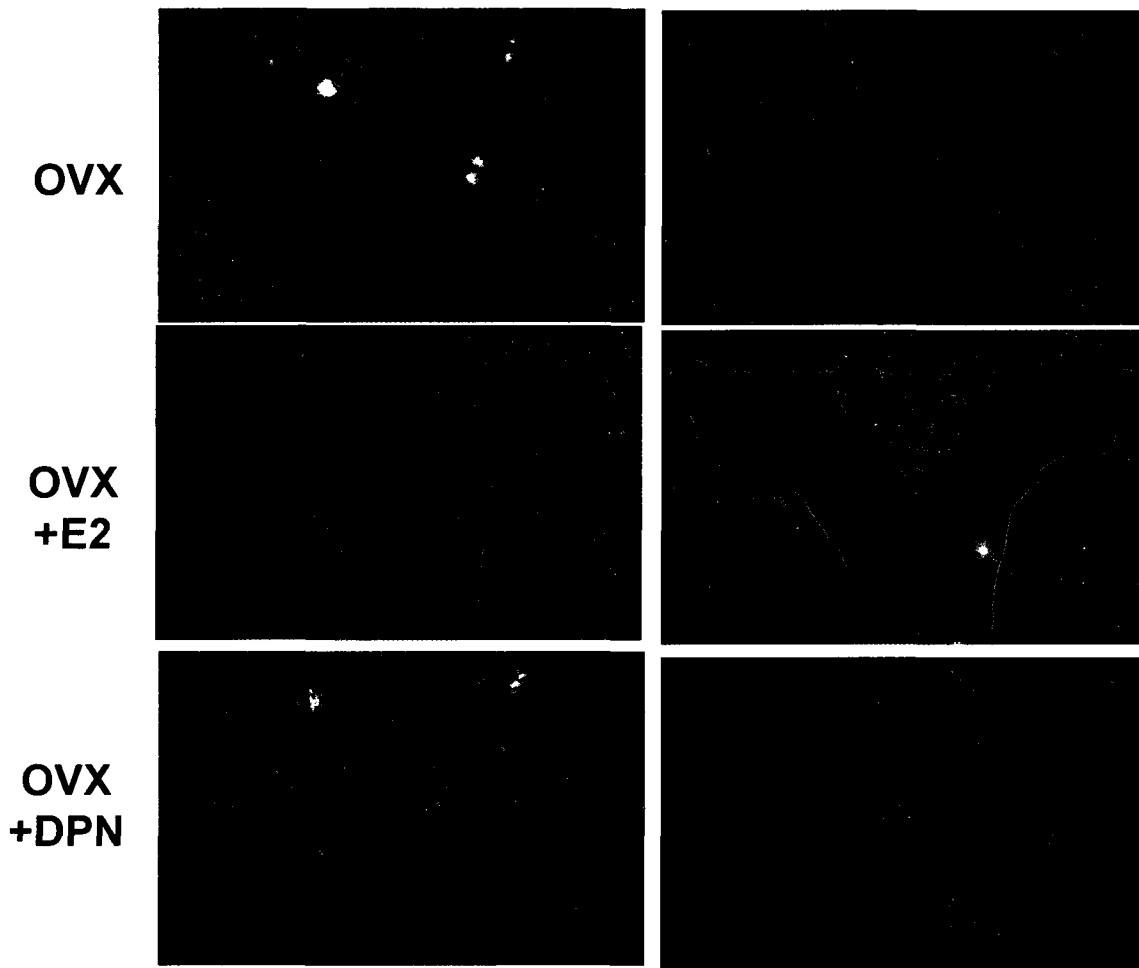
(A) Aortas from female apoE<sup>-/-</sup> mice (left) and HSP27<sup>0/e</sup>apoE<sup>-/-</sup> (right) mice subjected to ovariectomy (OVX, top row), OVX plus E2 replacement (OVX+E2, middle row) and OVX mice supplemented with DPN (OVX+DPN, bottom row).

(B) Atherosclerotic lesion quantification: lesion area is expressed as a percent of total arch area. n=7-9/group; \* p≤0.05.

(C) HSP27 Serum Levels Increase in Response to Estrogen and DPN Treatment. Serum HSP27 levels from HSP27<sup>0/e</sup>apoE<sup>-/-</sup> mice without hormone replacement therapy (OVX, solid line), with E2 treatment (OVX+E2, hatched line) and with DPN treatment (OVX+DPN, grey line) analyzed serially at baseline (before OVX), post-OVX (before pellet implantation), as well as 2 and 4 weeks following a HFD (expressed relative to baseline levels; n=7-9/group; \* p≤0.05).

**Figure 5.2**

**A**



**B**

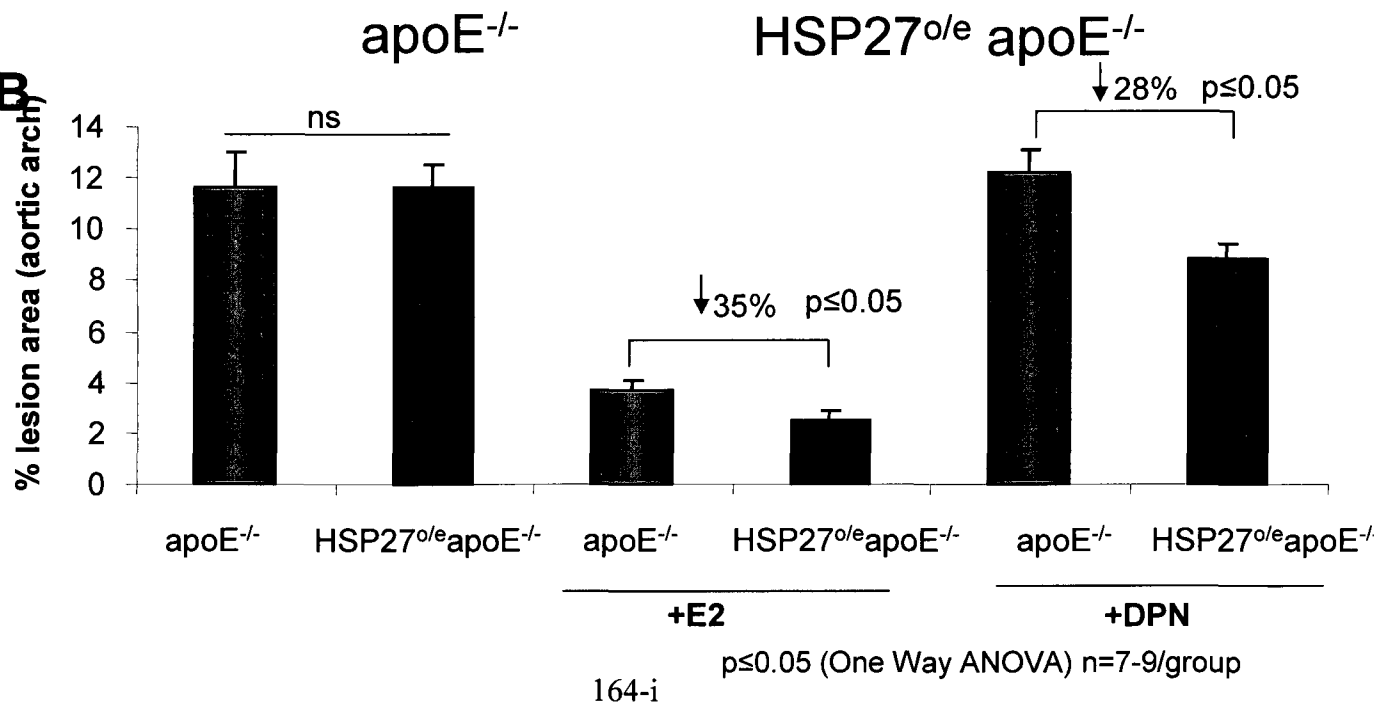
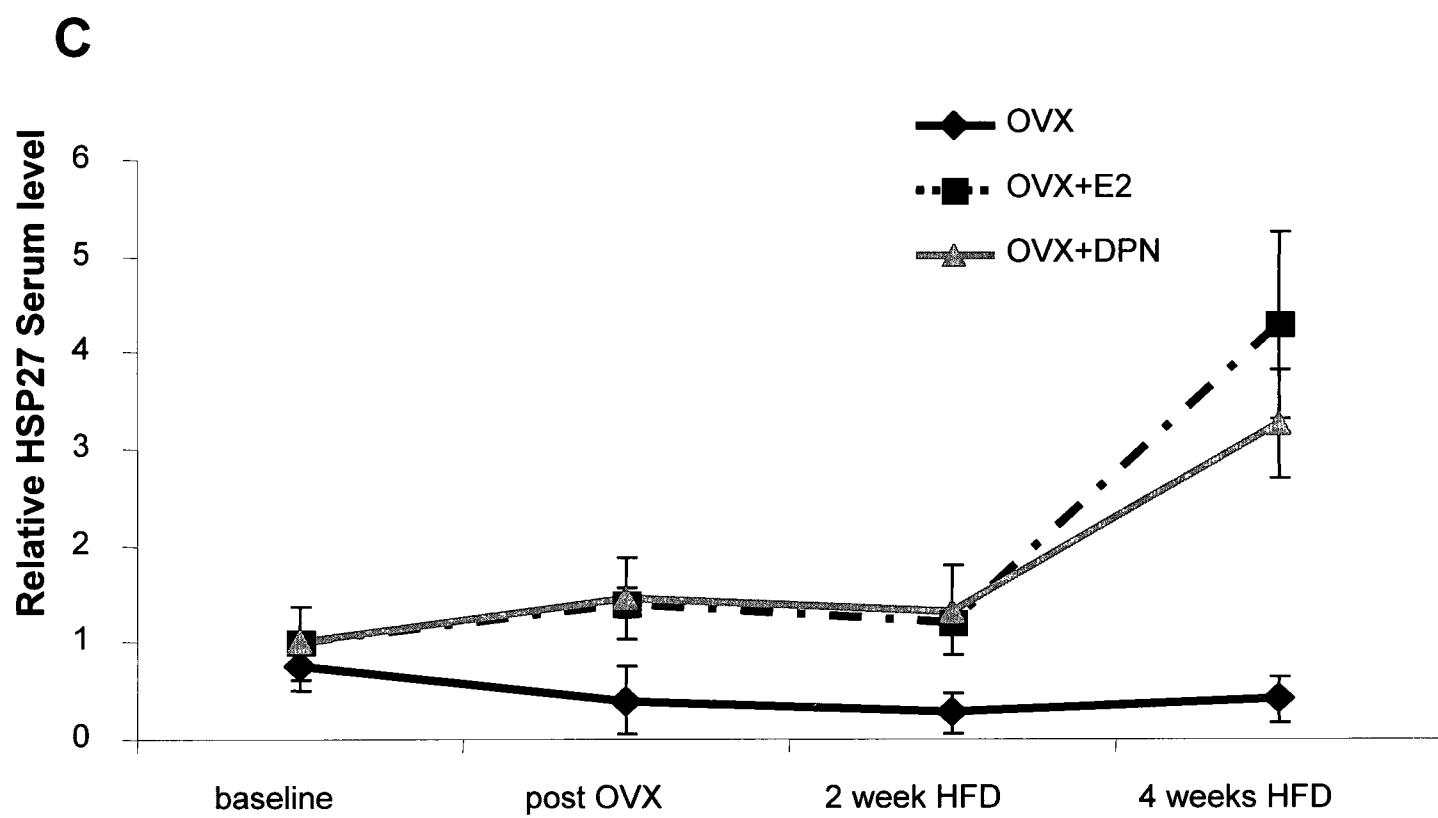


Figure 5.2



atherogenic diet, HSP27 levels were similar to baseline in ovariectomized mice that did not receive hormone replacement therapy (**Figure 5.2C**, solid line). Conversely, E2 supplementation caused a greater than 4-fold increase in HSP27 serum levels at 4 weeks compared to baseline (**Figure 5.2C**, hatched line;  $p \leq 0.05$ ) – an effect that was also observed with DPN treatment (**Figure 5.2C**, grey line).

## 5.6 Discussion

Previous studies demonstrated that estrogens are protective against atherogenesis.(5) In the current study we demonstrate that loss of ovarian hormones prevents the extracellular release of the atheroprotective factor, HSP27, in response to an atherogenic diet. Moreover, estrogenic replacement therapy not only produces a relative attenuation of atherogenesis in ovariectomized mice, but also promotes the release of HSP27 into the serum. *In vitro*, HSP27 release is more robust with modulation of ER $\beta$  compared to ER $\alpha$ , and *in vivo*, ER $\beta$  specific replacement therapy is able to recapitulate the rise in HSP27 levels, as well as promote atheroprotection. These data provide new mechanistic insights into how HSP27, a protein that was previously regarded as solely an intracellular chaperone protein, is released into the extracellular space in response to estrogen receptor modulation.

It is intriguing to consider that HSP27 may be the active mediator of the atheroprotective effects of estrogens - presumably via competitive antagonism of SR-A mediated LDL uptake (3,7). Looking downstream, we certainly note that HSP27 over-expression and supranormal HSP27 serum levels regulate the expression of a number of key genes involved in either

lesion development or regression.(4) The results of the current study align with parallel studies from our laboratory that outline how a different, more potent ER $\beta$  specific modulator (8 $\beta$ VE2) also produces a receptor dependent reduction in aortic lesion size (8). Studies are ongoing to determine whether estrogen receptor-mediated release of HSP27 can occur after a considerable delay post-ovariectomy and/or once atherosclerosis is already firmly established (e.g., analogous to the late initiation of HRT in post-menopausal women).

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## **6.0 DISCUSSION**

The data presented in this thesis brings forth important new mechanistic information regarding the role of estrogen in the vessel wall. Most importantly, we now understand that the outcome of estrogenic stimulation within the vasculature may depend on a multitude of factors, including which receptor is engaged, which ligand is used, and which co-factors are present. One of the major findings of this work is that estrogen acts through a novel ER $\beta$ -specific protein (HSP27) to attenuate the development of atherosclerosis. This implies that it is through the action of this co-factor rather than through the receptor itself that estrogen may exert its beneficial effects. Similarly, the expression of another important ER $\beta$  co-regulatory protein, NM23-H2, is lost with the progression of disease, again supporting the theory that the presence of these regulatory proteins is critical to maintenance of vessel wall homeostasis and the potential benefits of estrogen. The importance of these two proteins in the vessel wall, as well as the future of hormone replacement therapy, will be discussed in detail below.

### **6.1 NM23-H2: An important regulator of estrogen in the vessel wall**

#### **6.1.1 NM23-H2 Expression in the Vessel Wall**

NM23-H2 was discovered to be an ER $\beta$ -associated protein using a yeast-two hybrid screen of a cDNA library from human lung fibroblasts, which express high levels of ER $\beta$  (14). Although this interaction was confirmed using a variety of co-immunoprecipitation experiments using over-expression of both NM23-H2 and ER $\beta$ , it was not known if NM23-H2 was expressed by vascular cells in humans. It was shown that not only is NM23-H2 expressed at relatively high levels in both human SMCs and ECs *in vitro*, but in the normal

healthy vessel wall there is an abundance of NM23-H2 expression (see Manuscript #1, Chapter 2, **Figures 2.1 & 2.2**). However, as the vessel wall accumulates atherosclerotic plaque, the expression of NM23-H2 protein is strikingly diminished. This decrease in expression of an important ER $\beta$  regulatory protein could have detrimental consequences in the vessel wall in a number of ways. First, NM23-H2 is an activator of estrogen-induced gene transcription downstream of an ERE site and loss of this regulation could result in a change in gene expression patterns that affect normal vessel wall function. For example, estrogen is known to influence the expression of eNOS which, as discussed previously, is important for vasodilation and the maintenance of a healthy endothelium (23). Loss of regulation of this expression via a loss of co-regulatory molecules such as NM23-H2 could result in loss of eNOS regulation and hence unfavourable effects on the vessel wall. Second, NM23-H2 is a potent inhibitor of cell migration, primarily in the context of transformed tumor cells (10). We further demonstrated that estrogen can act synergistically with overexpression of NM23-H2 to enhance this anti-migratory effect (Manuscript #2, Chapter 3, **Figure 3.5E**). Therefore loss of NM23-H2 could result in an increase in cell migration in the vessel wall, possibly in both macrophage and SMCs, resulting in increased cell infiltration into the intimal area and ultimately increased plaque growth. As an activator of c-myc transcription, NM23-H2 has important roles in cell survival in response to a variety of stresses (2). One could speculate that a loss of NM23-H2 expression could result in increased apoptosis and/or necrosis within the vessel wall, that results in an expansion of the necrotic core area. Thus, due to its multifaceted nature, NM23-H2 influences a variety of important cellular processes in the vessel wall that are both dependent and independent of estrogen and potentially ER $\beta$ , and with progressive loss of NM23-H2, these processes can go unhindered.

It is not yet fully understood why the expression of NM23-H2 decreases as atherosclerosis progresses. One possibility is that the chronic inflammation within the vessel wall throughout the course of atherogenesis results in a downregulation of NM23-H2 expression. Within the atherosclerotic plaque, SMCs are devoid of NM23-H2 expression while ECs remain immunopositive (Manuscript #1, Chapter 2, **Figure 2.2B**). This is supported by the *in vitro* evidence that SMCs in culture lose their expression of NM23-H2 upon treatment with an inflammatory stimulus LPS (**Figure 2.3A**). Moreover, not only is NM23-H2 protein expression induced upon treatment with estrogen, but estrogen can rescue the loss in expression that occurs in response to inflammation (**Figure 2.3A**). As women age and circulating estrogen levels begin to decline, NM23-H2 expression (as well as other important genes regulated by estrogen, like HSP27) also begins to decline, which sets the stage for diseases like atherosclerosis. As the inflammation within the atherosclerotic plaque persists, and estrogen levels continue to decline, NM23-H2 expression is lost. Albeit not as striking as the loss of NM23-H2 with the progression of atherosclerosis, the expression of ER $\beta$  is also lost within the atherosclerotic plaque (**Figure 2.2D**). This loss of ER $\beta$  may be in part responsible for the observed reduced expression of NM23-H2, whose expression is induced upon stimulation with the ER $\beta$ -specific agonist DPN (**Figure 2.3A**). The observation that stimulation with both estrogen and DPN cause an increase in NM23-H2 protein expression may indicate that ER $\beta$  can induce feedback to increase expression of one of its co-regulatory molecules. Given that stimulation of ER $\alpha$  had no effect on NM23-H2 expression, this provides further evidence that ER $\beta$  in the vessel wall may be playing a protective role in part by inducing expression of protective proteins such as NM23-H2. This may prove to be advantageous in the context of hormone replacement therapy, which will more than likely

rely on the activation of ER $\beta$  rather than ER $\alpha$  in the vessel wall to prevent atherosclerosis. By finding ways to prevent the decline of NM23-H2 expression, or methods by which its expression can be rescued, this may provide the vessel wall with protection from injury via regulation of estrogen-mediated gene expression, increasing cell survival and inhibition of cell migration.

### **6.1.2 New insights into NM23-H2 Function**

After the initial discovery that NM23-H2 interacts with ER $\beta$  and the observation that NM23-H2 expression is diminished in atherosclerotic plaques, we made a number of key discoveries regarding this protein that may provide clues to how it functions. Using a double NM23-M1/M2 knock out (deletion of NM23-M2 alone is embryonic lethal) we discovered that deletion of these genes causes a complete absence of erythrocyte maturation, resulting in an excess of nucleated erythrocytes that cannot carry oxygen and an overall dysregulation of haematopoiesis. Coincidentally, parallel observations were made by another laboratory using mice deficient in NM23-M1 and –M2 (27). The dramatic effect of NM23 deletion on cell differentiation indicates that these proteins may play an important yet incompletely understood role in controlling the fate of certain hematopoietic cells. For example, in the healthy adult and during certain disease states involving hematopoietic cells (i.e. atherosclerosis development involving macrophages) the presence of NM23-H2 may be required for normal cell function and differentiation, and loss of this protein (as is the case with progressing atherosclerosis) may contribute to disease via dysregulation of cell fate. Should the loss of NM23-H2 expression favour commitment to a macrophage lineage, this could result in increased macrophage content within atherosclerotic plaques as NM23-H2

expression diminishes. Currently we are studying NM23-M1/M2<sup>+/-</sup> heterozygote mice which have reduced protein expression and are trying to determine how reduced levels of these proteins may alter the development of atherosclerosis.

## **6.2 HSP27: An Atheroprotective Protein**

Perhaps the most significant contribution of this doctoral work is the discovery that HSP27 is an atheroprotective protein that acts in concert with estrogen to reduce atherosclerotic burden. Over-expression of HSP27 in atherosclerosis-prone mice reduces aortic lesions by 35% - an effect that is dependent on the presence of estrogen (Manuscript #3, Chapter 4, **Figure 4.1**). Moreover, HSP27-dependent atheroprotection via estrogen receptor stimulation is preferentially modulated via ER $\beta$ , as treatment with DPN recapitulated the ~30% decrease in lesion area in HSP27<sup>oe</sup> mice (Manuscript #4, Chapter 5, **Figure 5.2B**). As serum HSP27 levels strongly and inversely correlated with atherosclerotic lesion size, with the highest HSP27 serum levels in the females corresponding to the smallest lesion areas ( $r^2=0.78$ , **Figure 4.2A&B**) we speculated that extracellular HSP27 is mediating the atheroprotective functions of this protein. We further solidified the key role for serum HSP27 levels in the prevention of disease by showing that ovariectomy abolishes the release of HSP27 into the serum, and hence abolishes the atheroprotective effects of HSP27 (**Figure 5.2C**). Conversely, treatment with either estrogen or DPN after ovariectomy causes HSP27 to be released once again into the serum, coinciding with reduced atherosclerotic lesion burden. Taken together these data strongly suggest that the serum HSP27 is primarily responsible for providing the atheroprotective benefit; however, the function of this protein in the vessel wall is multi-faceted, and could be mediated by both intra- and extracellular mechanisms.

Below is a discussion of the novel findings of how HSP27 functions in the vessel wall and what the future may hold for its use as a therapeutic target.

### 6.2.1 HSP27 in the Extracellular Space

As described above, the levels of HSP27 in the extracellular space were demonstrated to have a tight inverse correlation with the degree of atherosclerosis, particularly in female mice ( $r^2=0.78$ ;  $p\leq 0.05$ ). The discovery that HSP27 is secreted from cells in an active manner via stimulation with atherogenic lipids and/or hormone is novel; although HSP27 had previously been reported to be present in the circulation, the question of why or how it got there had not been addressed (9,21,26). Another novel finding is that HSP27 in the extracellular space can bind the scavenger receptor-A (SR-A), a receptor known to play several very important roles in the uptake of atherogenic lipids and foam cell formation (25). HSP27 can bind SR-A and compete for binding with acLDL, which results in a reduction of acLDL uptake, reduced foam cell formation and subsequent downstream inflammatory cytokine release (**Figure 4.5**). Macrophages from HSP27<sup>o/e</sup> mice following a high-fat diet are less lipid-laden compared to non-overexpressing mice, paralleling *in vivo* the reduction in foam cell formation observed *in vitro* (**Figure 4.6**). This indicates that the presence of HSP27 in the serum could have profound consequences on foam cell formation in the vessel wall and may prevent the progression of atherogenesis. Overall, HSP27 in the extracellular space appears to be anti-inflammatory and protective, which makes HSP27 a unique heat shock protein with properties that are distinct from those observed with other heat shock proteins, namely HSP60 and HSP70. For example, HSP70 binds the cell membrane via toll-like receptors 2/4 (TLR2/4), activates NF $\kappa$ B and causes the up-regulation of TNF- $\alpha$  (3). In the context of

atherogenesis, extracellular HSP70 binds macrophages and acts to enhance inflammatory cytokine release in response to oxLDL (28). Likewise, HSP60 binds monocytes via CD14 to elicit a pro-inflammatory response, similar to what is employed by bacterial LPS (13,29). In situations of ischemic stress, HSP60 is secreted by cardiomyocytes via the exosomal pathway, which could have detrimental consequences and further tissue damage in response to this release (12). This pro-inflammatory behaviour of HSP60 and HSP70 is believed to be due in part to the immune recognition of their closely related bacterial HSPs (4,5). Immunization with HSP60 and building immune tolerance to this protein before the initiation of disease may prevent atherosclerosis by attenuating the inflammatory response to its release (19,30). It is unknown what the exact role for these other HSPs may be in atherosclerosis development or prevention, but unlike HSP27, their presence appears to favour rather than prevent disease (7).

## **6.2.2 Mechanisms of HSP27 Secretion**

As mentioned above, the fact that HSP27 was detected in the serum of both healthy individuals and those with atherosclerosis and/or cardiac ischemia prompted us to investigate the mechanism of its release. The HSP27<sup>0/e</sup> mice provided clues as to which conditions might promote this protein to exit the cell: serum levels of HSP27 were highest in female mice following a high-fat diet, implying estrogens and/or an atherogenic stimulus might induce the secretion of HSP27 from cells. Indeed, macrophages *in vitro* secreted HSP27 in response to both estrogen and acLDL, replicating what was observed *in vivo* (**Figure 4.4**). Upon further examination, we demonstrated that HSP27 employs the exosomal pathway (at least in part) for its release in response to estrogen (data not shown), much like what has

been reported for other HSPs (8,22). The lysosomal pathway might also be involved in the release of HSP27 in response to acLDL; again similar to what is observed for HSP70 (16). Therefore although HSP27 does not contain a signal sequence that sorts it to a traditional secretory vesicle (like the Golgi) it is actively released in response to specific stimuli, and is not simply released as a result of cell necrosis or apoptosis, as HSP27 over-expressing macrophages even had reduced apoptosis compared to non-overexpressing cells yet these cells actively release HSP27 (data not shown). Interestingly, of the two estrogen receptors, the one primarily involved in HSP27 secretion is ER $\beta$ , as demonstrated both by using a specific activator of ER $\beta$  (DPN) *in vitro* and *in vivo*, and localization of HSP27 and ER $\beta$  at the cell membrane by confocal microscopy (data not shown). These data therefore expand our understanding of how HSP27 may be released into the extracellular space, but also supports the hypothesis that ER $\beta$  is indeed a protective protein in the vessel wall, possibly through its ability to induce secretion of an important anti-atherogenic protein HSP27.

### 6.2.3 HSP27 and the Estrogenic Response

Not only was HSP27 demonstrated to be atheroprotective and anti-inflammatory both *in vivo* and *in vitro*, but its effects were demonstrated to be dependent upon estrogen and ER $\beta$  (**Figure 5.1** and **5.2**). Ovariectomized HSP27<sup>oe</sup> mice showed no protection from atherosclerosis when compared to their non-overexpressing littermates, whereas replacing estrogen in these mice recapitulated the previously observed ~35% reduction in HSP27<sup>oe</sup> lesions. These results are interesting on many levels. First, it unequivocally proves that HSP27-mediated atheroprotection in acute atherogenesis is dependent upon the presence of ovarian hormones such as estrogen, and the effect that was previously observed in intact



mice was neither an artifact nor related to confounding factors such as background strain, etc. Second, the fact that estrogen provides a profound 68% reduction in lesion size independent of HSP27 (i.e. in the non-overexpressing mice) demonstrates its power as an anti-atherogenic hormone and is consistent with previously reports of reductions in lesion size in estrogen-treated mice (6). However, it is important to note that HSP27 provides an *additional* 34% reduction in lesion area to the reduction already provided by estrogen alone. This implies that although HSP27 requires estrogen for the acute protection from atherosclerosis, it can provide protection above and beyond that provided by estrogen, making it an extremely attractive therapeutic target for hormone therapy in both men and women.

Another significant finding in this doctoral work is that ER $\beta$  appears to be the receptor primarily responsible for the release of and subsequent atheroprotective actions provided by HSP27. Treatment of ovariectomized mice with DPN again recapitulated the ~30% reduction in atherosclerotic lesion area seen in the presence of either endogenous or exogenous estrogen, coinciding with a significant increase in serum HSP27 levels. Hence, these results confirm that ER $\beta$  accounts for the majority of the secreted and hence atheroprotective HSP27 that is observed when estrogen is present. Moreover, these data may also explain previous observations in our lab that HSP27 and ER $\beta$  appear to interact in the cytosol and not in the nucleus (1). In addition, in macrophages HSP27 co-localizes with ER $\beta$  at the cell membrane in vesicle-like structures (data not shown). The role for membrane estrogen receptors is now emerging as a signaling pathway that may eventually be of equal importance as estrogen-mediated signaling in the nucleus that involves transcriptional

activation (11). HSP27 secretion in response to an estrogenic stimulus via ER $\beta$  present at the cell membrane demonstrates a role for these membrane receptors in prevention from disease.

#### **6.2.4 Intra- versus Extracellular HSP27 Action**

One of the most interesting and exciting discoveries made throughout this doctoral work is that HSP27 release into the extracellular space provides protection from atherosclerosis. This conclusion was drawn from the following observations: (i) that serum HSP27 levels strongly and inversely correlate with atherosclerotic lesion burden ( $r^2=0.78$ ); (ii) that in the absence of HSP27 secretion post-ovariectomy, there is no protection from atherosclerosis; and (iii) under conditions that promote HSP27 release (i.e. after treatment with estrogen or DPN) atheroprotection is restored. Given the evidence that HSP27 can bind SR-A and reduce foam cell formation, we can argue that serum extracellular HSP27 is capable of reducing atherosclerosis. However, we have yet to definitively prove that it is the action of the extracellular and not the intracellular HSP27 that is providing the protection. Studies are underway delivering purified recombinant human HSP27 to mice in order to test if atherosclerosis can be reduced in the presence of high serum HSP27. We do know that intracellular levels of HSP27 within the atherosclerotic lesions of male and female HSP27<sup>o/e</sup> mice are not substantially different, yet it is only the female mice (with high levels of *serum* HSP27) that are afforded the protection from disease.

#### **6.2.5 Other mechanisms of HSP27-mediated atheroprotection**

Of course there are a plethora of mechanisms by which HSP27 might be providing its atheroprotective effects, both inside and outside the cell. Our hypothesis is that it is through

the extracellular HSP27 binding of SR-A which results in reduced foam cell formation and smaller lesions. However, little is known about how the extracellular signals are communicated that results in this anti-inflammatory and anti-migratory phenotype. To gain insight into these mechanisms, lesions from HSP27<sup>o/e</sup>apoE<sup>-/-</sup> and apoE<sup>-/-</sup> mice were collected and the mRNA from these lesions subjected to expression array analysis. Although these studies are ongoing, there are a number of intriguing observations regarding HSP27<sup>o/e</sup> lesions that have emerged: (i) these lesions have lower expression of a number of key inflammatory molecules (such as cytokines and chemokine receptors) compared to non-overexpressing lesions that support our theory that HSP27 is anti-inflammatory; (ii) molecules that have important roles in preventing atherosclerosis (e.g. serpin A1E) (15) are upregulated in HSP27<sup>o/e</sup> lesions compared to apoE<sup>-/-</sup> lesions; (iii) the expression of NM23-H2 is up-regulated 2-fold in HSP27<sup>o/e</sup> lesions compared to apoE<sup>-/-</sup> lesions, confirming that high levels of NM23-H2 are associated with decreased lesion areas. These new and exciting data will certainly provide new insights into how HSP27 may function (either intra- or extracellular) to alter gene expression and prevent atherosclerosis.

### **6.3 The Future of Estrogen and Cardiovascular Disease**

We now appreciate that the role for estrogens in the cardiovascular system is complex, and yet these hormones clearly provide protection from the development of atherosclerosis. Although it was once believed that the use of HRT throughout menopause would provide the same cardiovascular health benefits as endogenous ovarian hormones, it is now understood that this is simply not the case. The question remains: why did HRT fail to show any benefit in the prevention of cardiovascular disease? New information concerning some of the major

cardiovascular HRT trials is beginning to emerge that may shed some light on these confusing outcomes. For example, although the WHI trial concluded that HRT resulted in an increased risk of adverse cardiovascular events, these women were considered of advanced age (mean age  $63.3 \pm 0.7$  years, ranging from 50 to 79 years) and many of them had been post-menopausal for many years before beginning treatment (18). These women likely had underlying cardiovascular disease that had been developing throughout their post-menopausal years that may have gone undetected. In support of the “timing hypothesis”, a follow-up of the WHI trial was conducted in younger women (mean age 55 years) and demonstrated that women randomized to HRT had significantly lower disease burden than women receiving placebo, with up to 61% reductions in coronary artery calcification in estrogen-treated women (17). Both our group and others show that in both humans and mice, as coronary artery disease (CAD) progresses, there is a loss of HSP27 expression within the plaque, a protein we now know has important protective functions (21,24,26). However, these protective functions of HSP27 are dependent upon its secretion into the extracellular space induced by estrogen or selective estrogen receptor modulation (**Figure 6.1**). We speculate that as CAD progresses and HSP27 (and other estrogen regulatory proteins like NM23-H2) expression is lost, hormone therapies are less likely to provide protective benefits than if initiated in the earlier stages of disease, i.e. when HSP27 expression is abundant. The explanation for the loss of HSP27 remains unclear; some groups believe that proteases present within the atheroma can cleave extracellular HSP27 and render it inactive (20,31). Regardless, the loss of this protein is associated with increased disease

**Figure 6.1: Proposed mechanisms of HSP27 and estrogen-mediated protection from**

**atherosclerosis.** In the presence of estrogen (e.g. in younger pre-menopausal women)

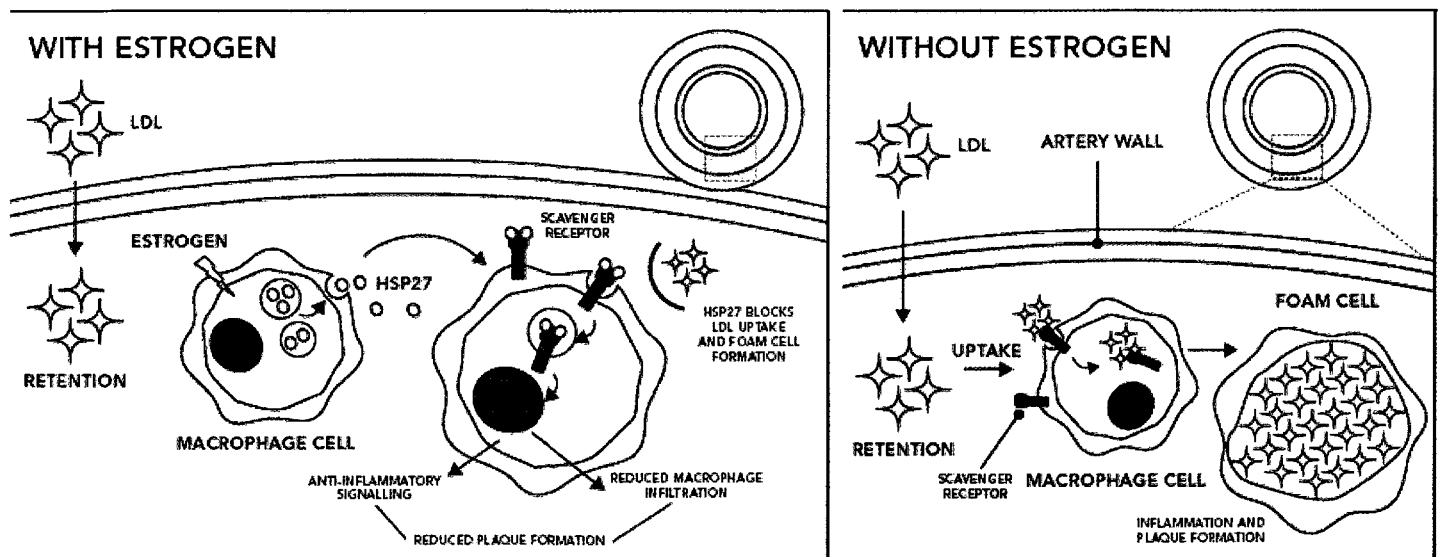
HSP27 secretion is induced, where it can bind the SR-A and prevent foam cell formation and

hence atherosclerosis progression. In the absence of estrogen (e.g. after menopause) there is

no HSP27 secretion into the extracellular space and thus no protection from foam cell

formation.

**Figure 6.1**



burden, potentially tempering the potential benefits of HRT. The future of hormone therapy for the treatment and prevention of cardiovascular diseases lies with the development of a targeted, specific estrogen receptor modulator that does not affect reproductive tissues like the breast and uterus, yet maintains estrogen-like effects on the vessel wall that stimulate favourable responses to prevent disease and maintain health (**Figure 6.2**).

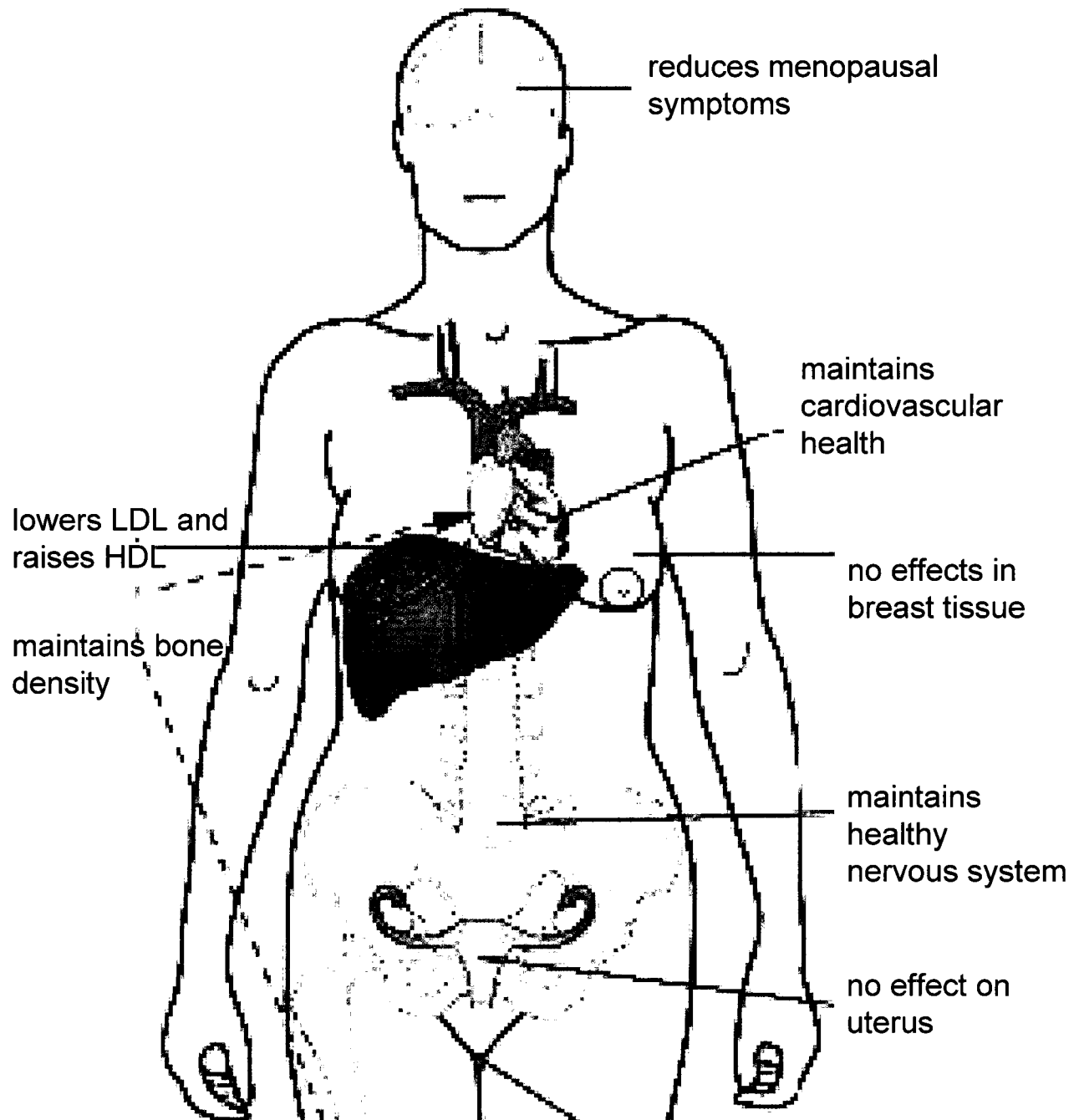
#### **6.4 CONCLUSION**

In conclusion, this doctoral thesis demonstrates a novel role for ER $\beta$ -associated proteins HSP27 and NM23-H2 in the prevention of atherosclerosis that may represent the missing link in our understanding of how estrogens mediate their protective effects in the vessel wall (**Figure 6.3**). Presented here are novel mechanisms of important estrogen regulatory proteins whose expression is lost as atherosclerosis progresses, and in the case of HSP27, can attenuate disease when expression levels remain high. The data supports a role for the targeted activation of ER $\beta$  within the vessel wall, as this receptor is capable of exerting many of the benefits of estrogen without the detrimental effects on reproductive organs. It also highlights the importance of how understanding the intricate molecular mechanisms of estrogen receptor action, at both the level of the receptor as well as its co-factors, can reveal complex and multifaceted roles of ovarian-derived hormones within the cell. The appreciation that the regulation of estrogenic activity depends on the presence of co-factors like HSP27 and NM23-H2 may lead to the development of improved therapies and may resurrect the use of hormone therapy for the treatment and prevention of cardiovascular diseases like atherosclerosis.

**Figure 6.2:** Design of an ideal selective estrogen receptor modulator (SERM). An ideal SERM would have positive effects on the bone, CNS, cholesterol and cardiovascular system, but would not affect breast and uterine tissues.

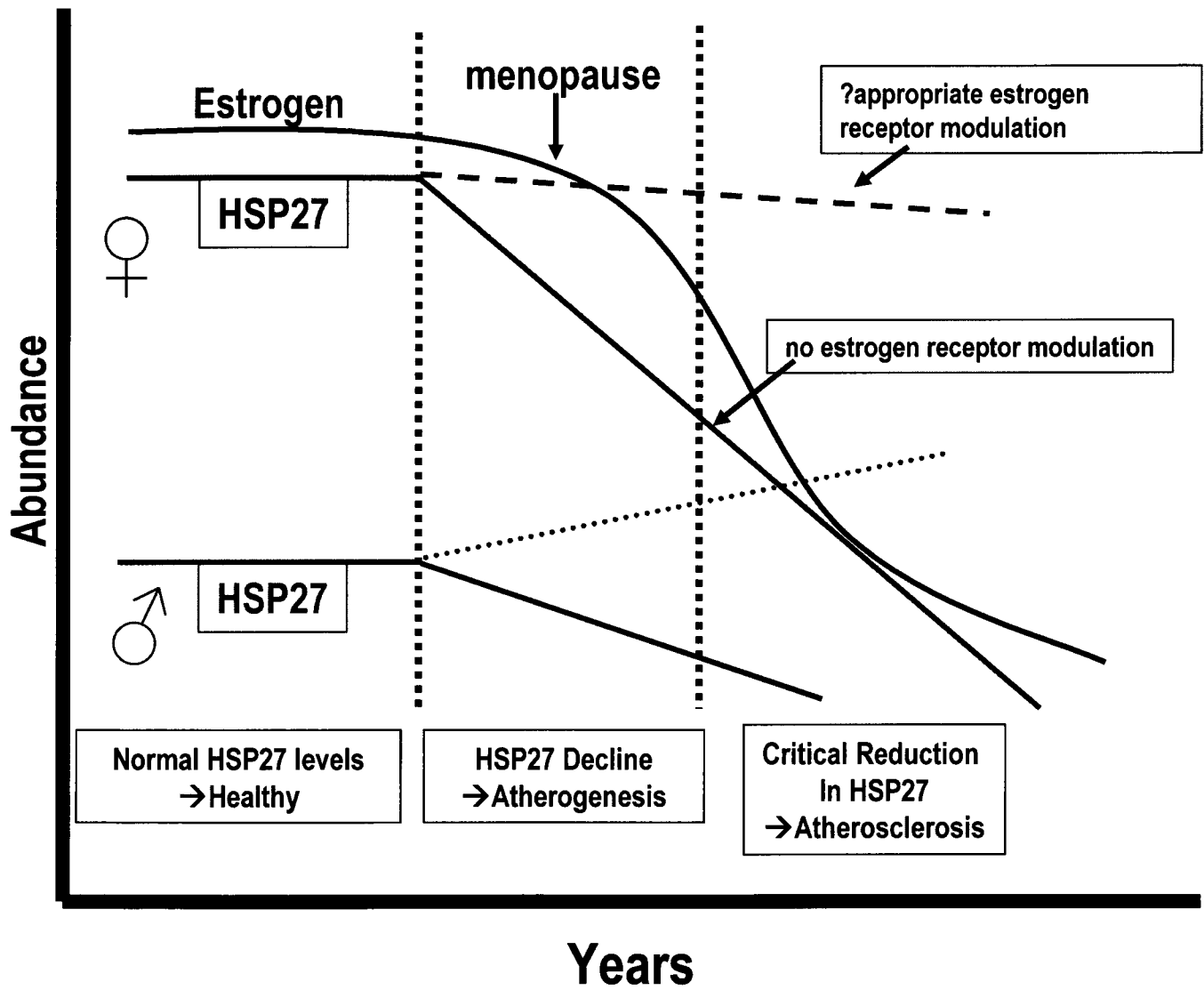


**Figure 6.2**



**Figure 6.3: Proposed interplay between HSP27 levels and atherosclerosis.** As estrogen levels begin to decline with increasing age, there is a concomitant decrease in HSP27 levels. Perhaps when this reaches a critical point, atherosclerosis can progress. However, with appropriate estrogen receptor modulation, speculation is that these levels can be rescued and atherosclerosis attenuated.

Figure 6.3



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## CURRICULUM VITAE

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### **Publications and Academic Accomplishments**

#### **Published refereed papers**

1. **“Extracellular Release of the Atheroprotective Heat Shock Protein 27 is Mediated by Estrogen and Competitively Inhibits acLDL Binding to Scavenger Receptor-A”** Katey Rayner\*, Yong X Chen\*, Melissa McNulty, Trevor Simard, Xiaoling Zhao, Dominic Wells, Jacqueline de BelleRoche, and Edward R O'Brien. (Circulation Research; Jul 18;103(2):133-41. Epub 2008 Jun 19)
2. **“NM23-H2, an Estrogen Receptor  $\alpha$  Associated Protein, Shows Diminished Expression with Progression of Atherosclerosis”** Katey Rayner, Yong Xiang Chen, Benjamin Hibbert, Dawn White, Harvey Miller, Edith H Postel, Edward R O'Brien. (Am J Physiol Regul Integr Comp Physiol. 2007 Feb;292(2):R743-50. Epub 2006 Aug 3.)
3. **“Discovery of NM23-H2 as an Estrogen Receptor  $\alpha$  Associated Protein: Role in Estrogen-Induced Gene Transcription and Cell Migration”** Katey Rayner, Yong-Xiang Chen, Benjamin Hibbert, Dawn White, Harvey Miller, Edith H. Postel and Edward R. O'Brien (J Steroid Biochem Mol Biol. 2007 Sep 16; [Epub ahead of print];108(1-2):72-81)
4. **“The Interaction and Cellular Localization of HSP27 and ER $\alpha$  are Modulated by 17 $\beta$ -Estradiol and HSP27 Phosphorylation”** Ashraf Said Al-Madhoun, Yong-Xiang Chen, Leila Haidari, Katey Rayner, William Gerthoffer, Heidi McBride and Edward R. O'Brien (Mol Cell Endocrinol. 2007 May 30;270(1-2):33-42. Epub 2007 Feb 13)
5. **“Modulation of estrogen signaling by the novel interaction of heat shock protein 27, a biomarker for atherosclerosis, and estrogen receptor beta: mechanistic insight into the vascular effects of estrogens.”** Harvey Miller, Stephanie Poon, Benjamin Hibbert, Katey Rayner, Yong Xiang Chen, Edward O'Brien (Arterioscler Thromb Vasc Biol. 2005 Mar;25(3):e10-4. Epub 2005 Jan 20).
6. **“Doxycycline alters vascular smooth muscle cell adhesion, migration and reorganization of fibrillar collagen matrices”** Christopher Franco, Bernard Ho, Diane Mulholland, Muzharul Islam, Katey Donaldson, Michelle Patricia Bendeck (Am J Pathol. 2006 May;168(5):1697-709).

#### **Book Chapters**

1. **“Foam Cell Formation During Atherogenesis”** Kathryn J. Moore and Katey Rayner; chapter contribution to: *Atherosclerosis: Cellular and Molecular Mechanisms*; Wiley-VCH; 2009.

### **Presentations as guest speaker**

1. Oral presentation given at the International Vascular Biology Meeting 2008 (IVBM 2008) in Sydney, Australia, Jun 1-5<sup>th</sup>, 2008.  
**TITLE: "Understanding the Atheroprotective Role of Heat Shock Protein 27"**  
Katey Rayner, Yong-Xiang Chen, Melissa McNulty, Trevor Simard, Xiaoling Zhao, Dominic Wells, Jacqueline de Belleruche, Jennifer Kreiger, Paromita Deb-Rinker, Edward O'Brien
2. Oral presentation given at the Canadian Cardiovascular Conference 2004 in Calgary, AB, for the Canadian Society for Atherosclerosis, Thrombosis and Vascular Biology oral session *The Vessel Wall*  
**TITLE: PuF Is a Novel Estrogen Receptor-Beta Associated Protein Involved in Vascular Cell Proliferation and Inflammation**  
K Donaldson-Rayner, H Miller, D White, YX Chen, E O'Brien

### **Published abstracts**

1. First author of abstract presented at the American Heart Association Scientific Sessions 2008 in New Orleans, Louisiana, Nov 9th-12th, 2008.  
**TITLE: "Estrogen Mediates the Extracellular Release of the Atheroprotective Heat Shock Protein 27 via Estrogen Receptor Beta"** Katey Rayner, Trevor Simard, Yong-Xiang Chen, Melissa McNulty, Xiaoling Zhao, Edward O'Brien. November 2008. Circulation Oct 2008 Supplement.
2. Co-author of abstract presented at the American Heart Association Scientific Sessions 2008 in New Orleans, Nov 9th-12th, 2008.  
**TITLE: "Over-expression of HSP27 Provides Chronic Atheroprotection: Reductions in Foam Cell Content and Inflammatory Responses"** (Oral Presentation). Y-X Chen, K. Rayner, P. Deb-Rinker, T. Simard, M. McNulty, X. Zhao, E.R. O'Brien. November 2008. Circulation Oct 2008 Supplement.
3. Co-author of abstract presented at the American Heart Association Scientific Sessions 2008 in New Orleans, Nov 9th-12th, 2008.  
**TITLE: "Selective Activation of Estrogen Receptor Beta Attenuates Atherogenesis and Represses Cholesterol-associated Immune-inflammatory Responses"** (Moderated Poster). K.Sun, X. Ma, K. Rayner, M. McNulty, F.D. Ramirez, T. Simard, B. Dhaliwal, Y-X Chen, B. Hibbert, E.R. O'Brien. November 2008. Circulation Oct 2008 Supplement.
4. Co-author of abstract presented at the American Heart Association Scientific Sessions 2008 in New Orleans, Nov 9th-12th, 2008.  
**TITLE: "The Atheroprotective Effects of HSP27 are Mediated by the Differential Expression of Multiple Inflammatory Genes"** (Oral Presentation). P. Deb-Rinker, Y-X Chen, K. Rayner, J. Kreiger, M. McNulty, D.Jurgens, N. Bahramifarid, X. Zhao, E.R. O'Brien. November 2008. Circulation; Oct 2008 Supplement.
5. Co-author of abstract presented at the American Heart Association Scientific Sessions 2008 in New Orleans, Nov 9th-12th, 2008.  
**TITLE: Glycogen Synthase Kinase 3 Beta Inhibitor Coated Stents Inhibit Neointimal Formation and Enhance Re-endothelialization Through Improved Endothelial Progenitor Cell Recruitment and Function"** (Oral Presentation). X. Ma, B. Hibbert, B. Dhaliwal, K. Rayner, Y-X. Chen, J. Sun, X. Zhao, T. Seibert, J. Raizman, E.R. O'Brien. November 2008. Circulation; Oct 2008 Supplement.

6. First author of an abstract presented as a poster at the ATVB Annual Conference 2008 in Atlanta, GA. April 15-18<sup>th</sup>, 2008.  
**TITLE: “Understanding the Atheroprotective Role of Heat Shock Protein 27”**  
 Katey Rayner, Yong-Xiang Chen, Melissa McNulty, Trevor Simard, Xiaoling Zhao, Dominic Wells, Jacqueline de Belleruche, Jennifer Kreiger, Paromita Deb-Rinker, Edward O'Brien
  
7. First author of an abstract presented as a poster at the American Heart Association Scientific Sessions 2007 in Orlando, FL, Nov 4-8<sup>th</sup>, 2007.  
**TITLE: Overexpression of the estrogen receptor beta associated protein, Heat Shock Protein 27, is Sex Specific and Atheroprotective**  
 Katey Rayner, Yong X Chen, Melissa McNulty, Trevor Simard, Xiaoling Zhao, Jacqueline deBelleRoche, Dominic Wells, and Edward R O'Brien  
 (*Circulation* Volume 116, Issue 16 Supplement; October 16, 2007)
  
8. Co-author of an oral presentation at the American Heart Association Scientific Sessions 2006 in Chicago, IL, Nov 12-15<sup>th</sup>, 2006.  
**TITLE: Heat Shock Protein 27: Influence on Endothelial Cell Homeostasis and Correlation with Coronary Artery Disease**  
 Yong-Xiang Chen, Katey Rayner, Trevor Simard, Daniel Ramirez, Edward O'Brien  
 (*Circulation* Volume 114, Issue 18 Supplement; October 31, 2006)
  
9. First author of poster abstract presented the Keystone Symposium entitled “Hormonal Regulation of Tumorigenesis” held in Monterey, CA, February 20-25<sup>th</sup>, 2005.  
**TITLE: Discovery of the Association of NM23-H2, a Metastasis Suppressor Candidate Protein, and Estrogen Receptor  $\beta$ : Potential Protective Role in Atherosclerosis**  
 Katey Rayner, Yong-Xiang Chen, Benjamin Hibbert, Dawn White, Harvey Miller, Edith H. Postel and Edward R. O'Brien
  
10. First author of poster abstract presented at the International Vascular Biology Meeting (IVBM 2004), June 1-5<sup>th</sup>, 2004, Toronto, ON.  
**TITLE: PuF, A Novel Estrogen Receptor-beta Associated Protein, Is Involved In Vascular Proliferation and Inflammation**  
 Katey Donaldson, Harvey Miller, Dawn White, Yong-Xiang Chen, Ed O'Brien  
 (*Cardiovascular Pathology* Volume 13, Issue 3, May 2004.)
  
11. Co-author of an abstract presented at the International Vascular Biology Meeting (IVBM 2004), June 1-5<sup>th</sup> 2004, Toronto, ON.  
**TITLE: The Effects of Doxycycline on Smooth Muscle Cell Adhesion and Aggregation**  
 Bernard KS Ho, Katey Donaldson, Diane Mulholland, Michelle P Bendeck  
 (*Cardiovascular Pathology* Volume 13, Issue 3, May 2004.)
  
12. Co-author of an abstract presented at the International Vascular Biology Meeting (IVBM 2004), June 1-5<sup>th</sup> 2004, Toronto, ON.  
**TITLE: The MMP inhibitor doxycycline prevents smooth muscle cell contraction of three-dimensional collagen gels.**  
 Christopher D Franco, Katey Donaldson, Michelle P Bendeck  
 (*Cardiovascular Pathology* Volume 13, Issue 3, May 2004)

### **In Press Abstracts**

1. First author of an abstract to be presented at the XV International Symposium on Atherosclerosis June 14th-18th, Boston MA.  
**TITLE: “Heat shock protein 27 atheroprotection is mediated by estrogen receptor beta”**  
Katey Rayner, Jianfeng Sun, Melissa McNulty, Yong Xiang Chen, Trevor Simard, Xiaoling Zhao, Edward O’Brien.

### **Patents/ Intellectual Property**

1. TITLE: “**USE OF HEAT-SHOCK PROTEIN 27 FOR CARDIOVASCULAR DISEASE PREVENTION AND TREATMENT**”

Patent number: PAT 4339-2

Date of Issue/Application: August 10, 2008

Inventors: Katey Rayner, Yong Xiang Chen, Xiaoli Ma, Edward O’Brien

Country Issued: United States

Impact/Relevance: Prevention and treatment of vascular disease.

## Rapid Communication

### Modulation of Estrogen Signaling by the Novel Interaction of Heat Shock Protein 27, a Biomarker for Atherosclerosis, and Estrogen Receptor $\beta$

#### Mechanistic Insight Into the Vascular Effects of Estrogens

Harvey Miller, Stephanie Poon, Benjamin Hibbert, Katey Rayner, Yong-Xiang Chen, Edward R. O'Brien

**Objective**—We sought to discover proteins that associate with estrogen receptor beta (ER $\beta$ ) and modulate estrogen signaling.

**Methods and Result**—Using a yeast 2-hybrid screen, we identified heat shock protein 27 (HSP27) as an ER $\beta$ -associated protein. HSP27 is a recently identified biomarker of atherosclerosis that is secreted at reduced levels from atherosclerotic compared with normal arteries. In vitro protein-binding assays confirmed the specific interaction of HSP27 with ER $\beta$  and not ER $\alpha$ . HSP27 expression was absent in coronary arteries with complex atherosclerotic lesions. Interestingly, HSP27 expression was also absent in 60% of coronary arteries from young males and females ( $27 \pm 6.5$  years) with normal histology or nonobstructive fatty streaks/atheromas. Moreover, the absence of HSP27 in these normal or minimally diseased arteries coincided with the loss of ER $\beta$  expression. Only 35% of arteries showed coexpression of HSP27 and ER $\beta$ . Relative to controls, estradiol-mediated transcription was reduced 20% with overexpression of HSP27 and increased 44% when HSP27 protein levels were reduced with HSP27 siRNA.

**Conclusions**—HSP27, an ER $\beta$ -associated protein, shows attenuated expression with coronary atherosclerosis and modulates estrogen signaling. (*Arterioscler Thromb Vasc Biol.* 2005;25:e10-e14.)

**Key Words:** atherosclerosis ■ hormones ■ receptors ■ signal transduction ■ women

There is a sex bias in the prevalence of cardiovascular morbidity and mortality that favors women until menopause; thereafter, this difference is lost.<sup>1</sup> Although a plausible explanation for this epidemiological distinction is the presumed vasculoprotective effect of ovarian hormones, randomized primary and secondary prevention trials involving hormone replacement therapy not only are nonconfirmatory but also document ill effects.<sup>2–4</sup> Estrogens act via at least 2 receptors that are expressed in the vessel wall (ER $\alpha$  and ER $\beta$ ), although there is increasing evidence that receptor-associated proteins play a critical role in determining the biological responses to ligand-dependent and ligand-independent signaling.<sup>1,5–9</sup>

We hypothesized that coregulators of ERs may modulate estrogen signaling in vascular tissues. In this article, we report that heat shock protein 27 (HSP27), a recently reported potential biomarker of atherosclerosis, specifically interacts with estrogen receptor beta (ER $\beta$ )—the receptor isoform that

shows transient mRNA overexpression early after vascular injury.<sup>10–12</sup> Using a differential proteomic screening approach, HSP27 secretion levels from human carotid plaques were found to be markedly diminished compared with normal arteries.<sup>10</sup> Moreover, circulating blood levels of HSP27 were decreased in patients with carotid atherosclerosis relative to healthy subjects. We now demonstrate that expression of HSP27 diminishes as the stage of coronary atherosclerosis progresses and that HSP27 is capable of regulating estrogen mediated transcription in vitro.

#### Materials and Methods

Briefly, our studies involve 4 major components: (1) the use of a yeast 2-hybrid screen to identify proteins that associate with the unique A/B domain of ER $\beta$ ; (2) the in vitro confirmation of the association of HSP27 with ER $\beta$  and not ER $\alpha$ ; (3) the demonstration of HSP27 expression in human coronary arteries; and (4) determination of the effect of modulating HSP27 levels on estrogen

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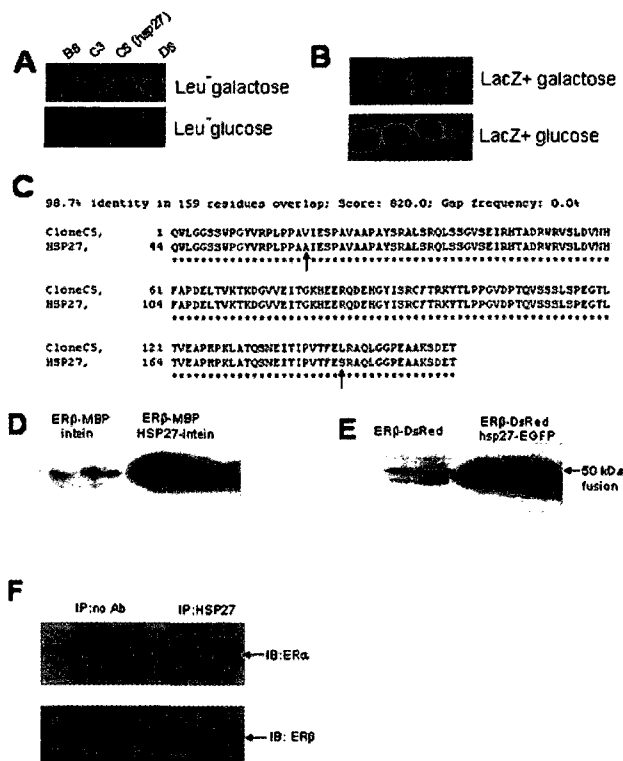
From University of Ottawa Heart Institute, Ontario, Canada.

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**Figure 1.** Identification of HSP27 as a novel ERβ associated protein. **A**, Yeast were transformed with an ERβ–LexA DNA binding domain and cDNA clones from the library fused to an activation domain before being selected for growth on galactose (top panel) and glucose (bottom panel) in the absence of leucine (Leu<sup>-</sup>). **B**, β-galactosidase activity was assessed on galactose (top) or glucose (bottom). **C**, Positive clone C5 was sequenced and identified as hsp27. Clone C5 shares 98.7% protein sequence identity with HSP27. **D**, Maltose-binding protein assay showing the interaction between the ERβ–MBP fusion product with intein protein alone and HSP27–intein fusion product, using antibodies specific for intein. **E**, Coimmunoprecipitation performed using HeLa cells transfected with ERβ–DsRed construct alone or ERβ–DsRed plus hsp27–EGFP. An antibody to DsRed was used for immunoprecipitation, and an antibody to HSP27 was used for immunodetection. **F**, Coimmunoprecipitation of HeLa cells transfected with an ERα expression vector (top panel) or an ERβ expression vector (bottom panel). For coimmunoprecipitation (IP), either no antibody (no Ab) or a HSP27-specific antibody was used. Both ERα-specific and ERβ-specific antibodies were used for immunoblotting (IB).

signaling. For a detailed account of the methodologies used in this article, please see <http://atvb.ahajournals.org>.

## Results

### HSP27 Interacts Specifically With the A/B Domain of ERβ

Using a yeast 2-hybrid screen, we identified previously unreported protein interactions with the ERβ. We selected the A/B domain of ERβ as our “bait” because it shares only 30% sequence identity with ERα. Dual-reporter gene activity (LEU2 and β-galactosidase) identified 77 positive screening results from a human lung fibroblast cell line cDNA library that contained  $5.3 \times 10^6$  clones. Restriction enzyme analysis identified duplicate clones, and the unique clones were sequenced. Because the A/B domain of ERβ contains the

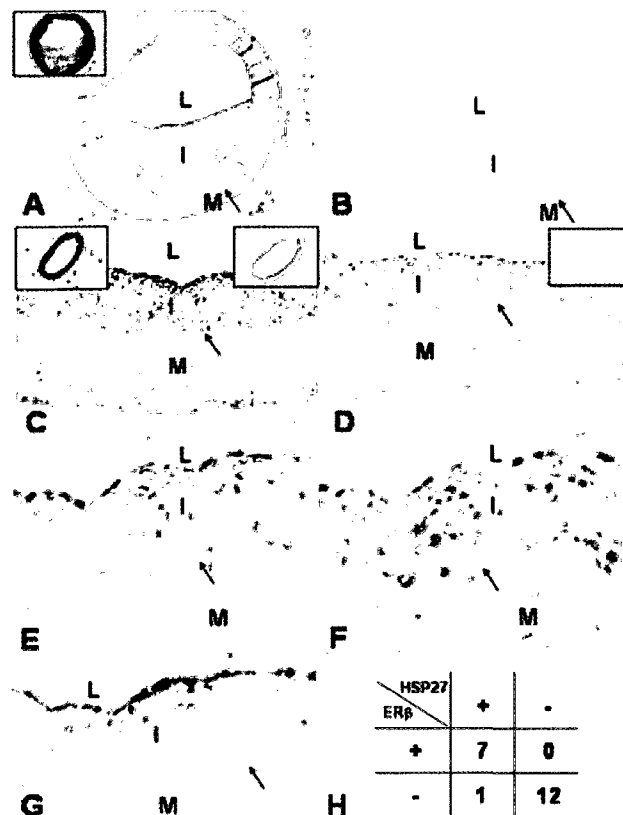
transcriptional activation function AF1, we ensured that selection gene expression was dependent on the interaction with the candidate proteins and not simply caused by auto-activation. Discounting ferritin and ribosomal proteins, which are commonly reported false-positives (<http://www.fccc.edu/research/labs/golemis/>), 3 unique clones remained: B8, D6, and HSP27 (BC073768; Figure 1A and 1B). All screens were repeated in triplicate to ensure the validity of the interactions. The HSP27 clone shared 98.7% sequence identity with the HSP27 protein sequence in the open reading frame (Figure 1C).

To confirm our yeast 2-hybrid findings, we used 2 separate *in vitro* assays. First, a fusion protein pull-down assay was performed using maltose binding protein (MBP) column precipitation. Fusion proteins of ERβ–MBP and HSP27–intein were expressed in bacteria, and then cell lysates were isolated. The ERβ–MBP protein was immobilized in a column before either HSP27–intein or nonfused intein cell lysates were run through the column. The retained product was then isolated and analyzed by Western blot with an intein-specific probe. With the empty intein construct, minimal protein was retained in the column, indicating that intein alone interacted only weakly with the ERβ–MBP product (ie, background; Figure 1D). However, the HSP27–intein fusion construct resulted in much darker band, consistent with a greater retention of protein and a strong HSP27 and ERβ interaction. Second, coimmunoprecipitation of the 2 proteins was performed. HeLa cells were transfected with either an ERβ–DsRed fusion construct (alone) or both ERβ–DsRed and HSP27–enhanced green fluorescent protein (EGFP) fusion protein constructs. An anti-DsRed antibody was used to immunoprecipitate proteins that bound to ERβ, and isolated proteins were analyzed by Western blot analysis using an HSP27 specific antibody. In HeLa cells transfected with the ERβ construct alone a weak band was observed—consistent with nonspecific binding (Figure 1E). In HeLa cells cotransfected with both the ERβ and HSP27 fusion protein constructs, the 50-kDa HSP27–EGFP fusion protein was immunoprecipitated, thereby indicating a specific interaction between ERβ and HSP27.

To ensure that HSP27 specifically interacted with ERβ and not ERα, coimmunoprecipitation of HeLa cell lysate from cells transfected with either an ERα or an ERβ expression vector was performed with an anti-HSP27 antibody. The precipitate was then analyzed by Western blot with both ERβ-specific and ERα-specific antibodies. The anti-HSP27 antibody clearly immunoprecipitated ERβ but failed to demonstrate an appreciable signal above background levels for ERα (Figure 1F).

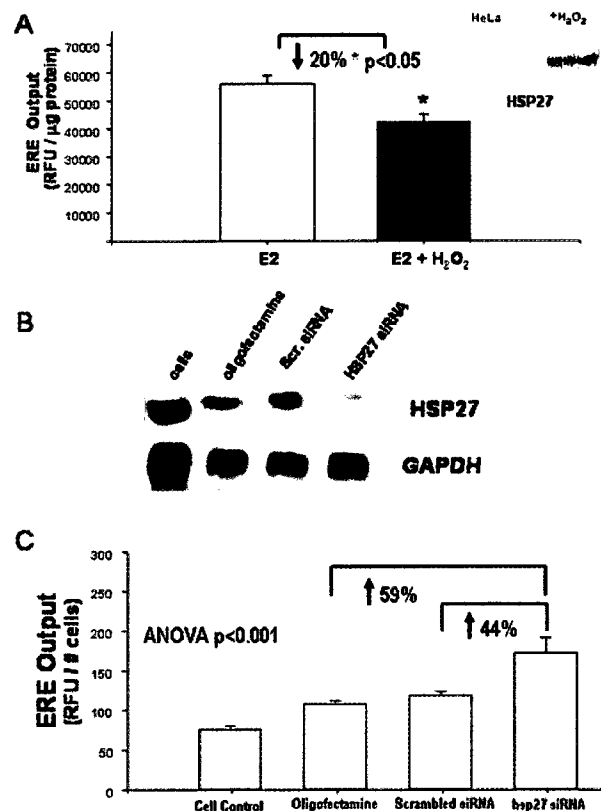
### HSP27 Is Coexpressed With ERβ in the Coronary Arteries of Young Individuals

The expression of ERβ and HSP27 was examined in coronary arteries with advanced atherosclerosis, as well as the proximal left anterior descending of 20 young human subjects who died of noncardiovascular causes (14 men and 6 women; age:  $27 \pm 6.5$  years). HSP27 expression was absent in those arteries with advanced atherosclerotic lesions (Figure 2A and 2B). However, in coronary arteries with benign intimal hyperpla-



**Figure 2.** Expression of HSP27 in human coronary arteries. Photomicrographs of human coronary artery cross-sections: advanced atheroma with necrotic cholesterol-laden intimal core labeled for (A)  $\alpha$ -smooth muscle actin and (B) HSP27 (magnification  $\times 100$ ; L indicates lumen; I, intima; M, media). HSP27 is absent in this atherosclerotic artery. Cross-section of a benign intimal thickening in the coronary artery of a young individual free of atherosclerosis, immunolabeled for  $\alpha$ -smooth muscle actin (C) and HSP27 (D) (magnification  $\times 400$ ). Top left insert of (A) and (C) show Movat pentachrome-stained slides; top right insert of (C) and (D) shows immunolabeling with anti-smooth muscle  $\alpha$ -actin antibody; magnification  $\times 40$ . Immunodetection of ER $\beta$  (E) HSP27 (F) and von Willebrand factor (vWF) (G) in normal coronary artery (magnification  $\times 1000$ ). Hematoxylin nuclear counterstain; brown reaction product for positive immunolabeling. The chart (H) displays the frequency of immunolabeling for ER $\beta$  and HSP27 in the coronary arteries of young individuals free of complex atherosclerotic disease. Apart for one exception (5%), arteries were either both immunonegative (60%) or both immunopositive (35%) for ER $\beta$  and HSP27.

sia or nonobstructive, pathological fatty streaks and/or atheromas (Stary type I and type III lesions, respectively), we noted variable expression of both HSP27 and ER $\beta$  (Figure 2C to 2F). The endothelium was present in all arteries, thereby discounting the possibility that false-negative immunolabeling results were the result of a denuded endothelium (Figure 2G). In these arteries, the following relationships were noted for the expression of HSP27 and ER $\beta$ : 7 of 20 (35%) were immunopositive for both, whereas 12 of 20 (60%) were negative for both (Figure 2H). Only one artery was immunopositive for HSP27 but lacked immunodetectable ER $\beta$ . No histological features were predictive of HSP27 or ER $\beta$  expression in these arteries; however, on serial tissue slides the expression of HSP27 and ER $\beta$  in luminal endothelial cells was clearly related (eg,  $R=0.94$ ,  $R^2=0.88$ ;  $P<0.0001$ ).



**Figure 3.** HSP27 and ERE signaling. HeLa cells stably transfected with an ERE-EGFP reporter construct were used to test the effects of elevated and decreased levels of HSP27 on estrogen signaling. All cells were treated with 100 nM 17 $\beta$ -estradiol. A, H<sub>2</sub>O<sub>2</sub> treatment to induce upregulated expression of endogenous HSP27 (inset Western blot) and resulted in a 20% decrease in ERE reporter output (relative fluorescence unit [RFU]; normalized to  $\mu$ g of total cell protein; \* $P<0.05$ ;  $n=12$ /group). B, Use of siRNA to HSP27 dramatically reduced HSP27 levels relative to scrambled (nonsense) oligomers and oligofectamine alone. GAPDH loading of each lane is shown in the lower row. C, The resultant decrease in HSP27 protein with siRNA treatment (B) produced increases in ERE output (RFU normalized to number of cells) of 44% and 59% relative to scrambled and oligofectamine, respectively (ANOVA:  $P<0.001$ , with  $P<0.05$  for the 2 noted comparisons).

### HSP27 Estradiol Mediated Transcriptional Activity

Finally, we examined the role of HSP27 in modulating the estrogen transcriptional activity in HeLa cells stably transfected with an estrogen response element (ERE)-driven EGFP reporter. First, we examined the effect of elevated HSP27 levels on estrogen signaling by inducing overexpression of endogenous HSP27 by treating the cells with H<sub>2</sub>O<sub>2</sub>, a form of oxidative stress (Figure 3A).<sup>13</sup> Induction of HSP27 overexpression (Figure 3A inset) caused a 20% decrease in 17 $\beta$ -estradiol transcriptional activity, as reflected by a decrease in relative fluorescence units normalized to the  $\mu$ g of protein per well, compared with control cells treated with 17 $\beta$ -estradiol but not H<sub>2</sub>O<sub>2</sub> ( $P<0.05$ ; Figure 3A). Second, we used HSP27-siRNA to reduce HSP27 protein levels in the same ERE reporter construct-transfected HeLa cells. Relative to oligofectamine or scrambled controls, siRNA dramatically reduced HSP27 protein expression (Figure 3B) and increased



ERE output 44% and 59% compared with scrambled (non-sense) oligomers and oligofectamine, respectively (Figure 3C; ANOVA  $P < 0.001$ , with  $P < 0.05$  for the mentioned comparisons). Taken together, these findings suggest that HSP27 is a corepressor of estrogen signaling mediated by ERβ.

### Discussion

Nuclear receptor-associated proteins are important determinants of the cellular response to ligand-dependent and ligand-independent steroid hormone signaling.<sup>1,9</sup> Because we are interested in the vascular effects of estrogens, we sought to identify possible coregulatory proteins of ERβ, the ER isoform that shows transient mRNA overexpression after vascular injury.<sup>11,12</sup> Using a yeast 2-hybrid screen, we discovered the association of HSP27, a recently recognized biomarker of atherosclerosis, with ERβ.<sup>10</sup> By way of various in vitro protein assays, we confirmed the interaction of HSP27 with ERβ but not ERα. Whereas Martin-Ventura et al recently noted decreased secretion of HSP27 in the supernatant of atherosclerotic carotid plaques compared with normal human arteries, these investigators did observe HSP27 immunopositive smooth muscle cells in atherosclerotic carotid endarterectomy specimens and normal mammary arteries. In contrast, we found an absence of HSP27 expression in coronary arteries with complex atherosclerotic lesions. There are several potential explanations for this discrepancy in the HSP27 immunolabeling results: (1) different arteries were examined (carotid versus coronary); (2) perhaps the complexity of the atherosclerotic lesions differed; and (3) we used a monoclonal anti-HSP27 antibody whereas Martin-Ventura et al used a polyclonal anti-serum to HSP27. Although immunolabeling has its limitations, we were intrigued to find either an absence or presence of both HSP27 and ERβ in all but 1 coronary artery from this population of young subjects. Whether the expression of these 2 proteins is linked requires further study. Interestingly, sex did not predict expression of ERβ or HSP27.

Heat shock proteins are highly conserved molecular chaperones that show upregulated expression in response to a range of cellular insults (eg, heat, oxidative stress, infection, cytokines) and play an active role in the stabilization and refolding of key intracellular proteins.<sup>14</sup> Although vascular smooth muscle and endothelial cells are known to express HSP27, the role of this protein in the vessel wall is only now beginning to be studied. Moreover, a number of studies report provocative associations between circulating heat shock protein levels or anti-heat shock protein antibodies and vascular disease.<sup>15</sup>

Even though it has been known for more than a decade that HSP27 is induced by estrogens and in some way associated with estrogen receptors in various cells (eg, breast and endometrial tumors, platelets), these studies were completed before the discovery of ERβ.<sup>16,17</sup> Our study is the first to report that HSP27 specifically associates with ERβ and acts as a corepressor of estrogen signaling. Coregulatory proteins confer milieu-specific responses to steroid hormone receptors and altered levels of these factors play important roles in

some diseases. For example, Gregory et al demonstrated that steroid receptor coactivators of the p160 family are expressed in the normal endometrium during the menstrual cycle but overexpressed in women with polycystic ovarian syndrome, and lead to poor reproductive function, endometrial hyperplasia, and cancer.<sup>18</sup>

Similarly, as a corepressor of estrogen signaling, HSP27 may play a role in atherogenesis. However, the critical question that needs to be resolved is whether HSP27 loss contributes directly to coronary atherogenesis or if it is merely a secondary phenomenon that follows the accumulation of an atherosclerotic plaque? Given that induction of HSP27 with herbimycin A reduces neointimal hyperplasia in rat carotid arteries subjected to balloon injury, it is attractive to postulate that the relative absence of HSP27 may in fact be an important mechanistic step in atherogenesis.<sup>19</sup> For example, because many important vascular growth factors and cytokines have an ERE, it is conceivable that unregulated estrogen mediated transcription of these factors might occur in the absence of HSP27 and foster atherogenesis.<sup>20</sup> Hence, knowledge of an individual's HSP27 "status," perhaps reflected by a simple blood test, may be predictive of atherogenesis and who should receive estrogen therapy, because the development of undesirable side effects (eg, venous thrombosis, malignancy) could be caused by loss of HSP27 regulation of estrogen-mediated transcription. With studies of HSP27 ongoing in various patient populations, the usefulness of this biomarker in clinical management will soon be clarified.

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