REGULATORY ROLES OF ENDOTHELIAL CELLS IN CANCER

By

Joseph W. Franses

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Signature of Author _____
Harvard-MIT Division of Health Sciences and Technology
May 16, 2011

Certified by: _____
Elazer R. Edelman, M.D.-Ph.D.
Thomas D. and Virginia W. Cabot Professor of Health Sciences and Technology, M.I.T.
Thesis Supervisor

Accepted by: _____
Ram Sasisekharan, Ph.D.
Edward Hood Taplin Professor of Health Sciences and Technology and Biological Engineering, M.I.T.
Director, Harvard-M.I.T. Division of Health Sciences and Technology

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on May 16, 2011 in Partial Fulfillment of the Requirements for the Degree
of Doctor of Philosophy in Biomedical Engineering

Advisor: Elazer R. Edelman, Thomas and Virginia Cabot Professor of Health Sciences and Technology, M.I.T.

Thesis committee chair: David A. Housman, Ludwig Professor of Biology, M.I.T.
Thesis committee:
1. Sangeeta N. Bhatia, Professor of Health Sciences and Technology and Professor of Electrical Engineering and Computer Science, M.I.T.
2. David T. Scadden, Gerald and Darlene Jordan Professor of Medicine, Harvard University

Abstract

This thesis describes the biochemical regulatory impact of endothelial cells, the cells that line all blood vessels, in cancer. Our work draws from concepts in vascular repair and tissue engineering and extends the view of tumor vessels from perfusing tubes to delivery platforms lined with potent paracrine regulatory cells. We focus on how the endothelial cells themselves regulate tumor biology in a state-dependent fashion.

We found that healthy endothelial cells inhibit cancer cell proliferation, invasiveness, and inflammatory signaling and that a defined perturbation of the healthy endothelial cell state – silencing of the gene encoding perlecan – causes loss of the invasion-inhibitory capabilities of endothelial cells by transcriptional upregulation of IL-6. The use of matrix-embedded endothelial implants enabled the effects in cell culture to be expanded and validated in animal models. Moreover, endothelial cells exposed to a pathologically activating and inflammatory culture environment, similar to endothelial cells exposed to the atherosclerotic milieu, were leaky and inflamed, with dysregulated proliferative and leukocyte binding properties. Unlike healthy endothelial cells, which suppress cancer cell proliferation and metastasis, these dysfunctional endothelial cells instead aggressively stimulated cancer cell inflammatory signaling and invasiveness, which correlated with stimulation of spontaneous metastasis when implanted as matrix-embedded cell implants adjacent to tumors. Fascinatingly we were able to identify markers of endothelial dysfunction, including reduction of endothelial perlecan expression, in human non-small cell lung carcinoma specimens.

The state-dependent impact of endothelial cells on cancer biology adds another element to stromal regulation of cancer and brings together a range of disciplines and disparate findings regarding vascular control of tumors. That healthy endothelial cells suppress and dysfunctional cells promote tumor aggression may help to explain undesired effects of therapies that target tumor blood vessels. The harnessing of tissue engineering to regulate vascular and cancer biology may motivate the development of innovative pharmacologic and cell-based therapies for cancer.

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CHAPTER 1: INTRODUCTION, BACKGROUND, AND SIGNIFICANCE

Abstract

Although the roles of endothelial cells in cancer have been primarily considered to be related to tumor perfusion, there is emerging appreciation of "angiocrine" regulation - adding stromal regulatory capabilities to the expanding list of endothelial functions in tumors. We posit that understanding the state-dependent paracrine regulatory paradigms established in vascular disease and repair will be critical for a deep understanding of tumor biology, as endothelial cells regulate diverse physiologic and pathophysiologic processes in all vascularized tissues. We now outline the historical developments that led to the appreciation of the paracrine regulatory functions of endothelial cells, summarize classical views of blood vessels and stroma in cancer, and attempt to merge these ideas to include the stromal regulatory endothelial cell as a critical regulator of cancer. The notion of the endothelial cell as a biochemical regulator of cancer state in constant dynamic balance with its tumor could impact diagnosis, prognosis and treatment of cancer. Such concepts might well explain the mixed results from anti-angiogenic cancer therapeutics and how certain drugs that improve vascular health correlate with improved cancer prognosis.

"In the time available I have been able to show you a little of the current knowledge of the morphology of [endothelial] cells which fifteen years ago were thought to form little more than a sheet of nucleated cellophane." Lord Florey [1].

Introduction

In the mid 19th century Rudolph Virchow [2] included "abnormalities of the blood vessel wall" as one of the critical elements of his classic triad defining propensity for clotting – the others being blood coagulability and flow disruption. We interpret this abnormality to mean endothelial dysfunction and seek to ascribe to Virchow the deepest insight as to the bioregulatory function of the endothelium, and extend the triad as risk not only of thrombosis but of all vascular pathologies. The current dogma holds that vascular health is synonymous with endothelial integrity and that disruption of endothelial health presages and contributes to vascular disease. We attribute Virchow's inclusion of mural abnormalities as early reference to the notion that the state of the endothelial cell determines if overlying blood will flow or clot, circulating leukocytes will adhere and transmigrate, underlying vessels will constrict or dilate and adjacent smooth muscle cells proliferate or regress.

As we continue to consider these issues in vascular biology, the analogy to tumor biology and its vascular dependence is obvious and intriguing. We now present our thoughts on the convergence of the biologies of vascular repair or injury with tumor control or spread, and especially how the use of matrix-embedded endothelial cells can

help to reveal complex regulatory mechanisms in physiology and disease. Attributing our thoughts to great minds of the past might make our current view seem great. Yet, today's perspectives took more than a century to evolve and even Virchow's original intent remains disputed [3]. Our present views will undoubtedly require continued refinement and cannot account for all that came before or include all potential perspectives. We hope that they will generate discourse and further investigation.

Vascular biology's origins

Functional studies of the vasculature originated with Ernest Starling in 1896 [4] and Ramon y Cajal (~1933), Edmund Cowdry, Alfred Kohn, and others [5]. Supported with a quantitative framework provided by John Pappenheimer [6] they surmised that the endothelium served primarily as a selectively permeable vascular lining. Examination of endothelial cell control of vascular tone [7-10], thrombosis [11-12], hyperplasia [13-14], and inflammation [15-17] was complemented by investigation of endothelial sensitivity to biomechanical stimuli, including shear stress, hydrostatic pressure, and circumferential strain [18-19].

Insight into the structural biology of the endothelium was made possible by great technical achievements linked to deep scientific insight – Morris Karnovsky's work on novel cytochemical investigations into intact vascular ultrastructure amongst the many important findings [20]. Florey cites this work in his tome on endothelial physiology – a work that was astonishingly prescient in its scientific implications and general perspectives regarding the synergistic progress of the science and enabling

technological innovations of vascular biology. Drugs that regulate clotting, blood pressure, cholesterol metabolism and heart failure and endovascular implants could not have been conceived of, developed or refined without deep understanding of vascular biology, and the use of these drugs and devices provided new means of probing physiologic systems. Detailed examination of endothelial cell biology was propelled further when Jaffe, Nachmann, Becker, and Minick [21] and Gimbrone, Cotran and Folkman [22] described the stable culture, identification and study of isolated endothelial cells. These landmark achievements summarized eloquently in [23] led in the decades hence to thousands of investigations into endothelial cell functions.

The endothelial cell, endothelium and vascular structure.

Large vessels are endothelial-lined tubes and like similar epithelial-lined tubes are comprised of a trilaminate architecture. The three vascular mural tunics interface with the lumen from within, the viscera from without, and contain a muscular layer in between. The innermost *tunica intima* contains endothelial cells and their underlying extracellular matrix (ECM) layer, the basement membrane, and in larger vessels vascular smooth muscle cells. Beneath the intima, separated by the internal elastic lamina, is the *tunica media*, with phalanges of smooth muscle cells separated into packets by fascia and connective tissue sheets. The densest of these sheets bounds the media – on the intimal side as the internal elastic lamina and on the interface with the *tunica adventitia* as the external elastic lamina. The adventitia contains nerves ("*vasa nervosum*," nerves of vessels), fibroblasts, extracellular matrix, and capillaries ("*vasa vasorum*," vessels of vessels). The capillaries, often little more than endothelial

cells and sparse supporting pericytes, comprise a second vascular network that runs as *vasa externa* parallel to the vessel. As the vessel wall thickens beyond a critical limit mural tissue requires additional perfusing vessels and a network of *vasa interna* becomes evident. The *interna* is connected to the *externa* via communicating capillaries. This thickness limit is reached always in larger species and in particular in atherosclerotic vessels. The arteriopathic lesions of atherosclerotic plaques [24] and catheter-induced intimal hyperplasia [25] are rich in and dependent upon vasa vasorum for their continued progression.

There are then two primary sources of endothelial cells in large vessels – those that reside at the lumen of the large vessel and those of the *vasa vasorum* that run parallel to and then course through the vessel wall, in a sense perpendicular to the wall. The large vessels' endothelial cells regulate and sense flow, interact with blood-borne elements [18-19], and modulate permeability. The capillary endothelial cells of the *vasa vasorum* are far more abundant and their ubiquity provides that every cell in the vessel wall is within two oxygen diffusion lengths from an endothelial cell. Every vascular cell is therefore under the potential regulatory control of an endothelial cell [26]. Since all tissues contain microvasculature, every cell in every tissue is under similar potential regulatory control.

Endothelial cells are ubiquitous, plastic, paracrine regulators of inflammation and macrovascular disease.

The power of endothelial-derived regulation resides not only in its interfacial position but also in the plasticity of the system. The same cells can respond differentially based on subtle alterations in microenvironmental cues and on their own phenotypic state [26]. For example, the density-dependence of endothelial regulatory phenotype modulates control of vascular smooth muscle hyperplasia [27-29], attraction and trafficking of leukocytes [30], and vascular smooth muscle [27-28] and even epithelial hyperplasia [31]. Healthy endothelium responds to increases in blood flow by releasing factors like nitric oxide that induce vasodilatation, while injured endothelial layers respond to the identical flow stimulus with the release of a countervailing set of factors that cause vasoconstriction. This latter response of macrovascular endothelium to increased fluid flow was termed paradoxical and formed the original definition of "endothelial dysfunction" [32]. The state-dependent endothelial regulatory phenotype has been extended to explain perivascular angiogenesis [33], hematopoiesis and thrombopoiesis [34-36], and most aspects of inflammation [37].

Thus, the endothelium responds to and regulates diverse physiologic and pathophysiologic processes. We classically have defined polar forms wherein dysfunctional endothelium stimulates disease, but quiescent endothelium suppresses disease and, hence, stimulates repair. Yet, it may very well be that endothelial cells can attain a range of states from pure quiescence to repair-inducing to activated, highly inflamed and disease-promoting. We posit that there are actually not two but rather

three fundamental states: dormant or quiescent, physiologically activated and reparative, and dysfunctionally activated and therefore disease-stimulatory. It is clear that atherosclerosis, and therefore coronary and peripheral arterial disease [18], kidney disease and uremia [38], diabetes mellitus and metabolic syndromes [39], rheumatoid arthritis [40], hypertension [41], pre-eclampsia [42], and many other pathologic states strongly correlate with endothelial dysfunction. All of these disease states share significant inflammatory and oxidative stress components, and endothelial dysfunction is a hallmark of and contributor to these diseases and can be used to classify disease severity [43]. That endothelial state could now explain diverse elements of tumor biology is intriguing.

Endothelial cell state and substratum interactions

Endothelial state is both manifested in and regulated by the composition of the underlying extracellular matrix. The basement membrane on which endothelial cells reside provide critical adhesion molecule ligand-binding sites [44]. The biochemical composition of the basement membrane serves as a depot for signaling molecules – some of which are themselves derived from extracellular matrix components – and growth factors that regulate endothelial and neighboring cells [45]. Turnover or degradation of basement membrane by matrix-digesting enzymes can cause profound changes in the local tissue environment [46]. It is not surprising then that particular extracellular matrix molecules can promote endothelial reparative capabilities – e.g. the heparan sulfate proteoglycan perlecan [47-48] – and modification or degradation of these molecules or increasing the activity of opposing molecules can promote

dysfunction. Destructive stimuli may therefore target both the cell and matrix components, and endothelial dysfunction can arise from targeted disease in either.

Embedding endothelial cells within porous biocompatible polymer three-dimensional scaffolds stabilizes endothelial phenotype by controlling cell-substratum interactions. Regulatory units of precise number and with controlled biosecretion patterns can be created in a range of forms for facile implantation in a variety of culture and animal models. Our laboratory has used such cellular devices to create a reproducibly reparative endothelium, and demonstrated the power of this engineered organ in many models of hyperplastic and inflammatory diseases [49-57]. Unlike injections of isolated cells that require significant time for homing and engraftment, cell accommodation, maturation, adhesion, etc., a matrix-embedded endothelial cell construct is immediately effective, behaves consistently and since its function is quantifiable before implantation the end result is therefore predictable. Regulatory effects and potency are sustained long after constructs erode and without generating a significant inflammatory or immune response. Allogeneic and even xenogeneic matrix-embedded endothelial cells placed perivascularly provide long-term inhibition of intimal hyperplasia following controlled vascular injury [33, 58-60] and inhibit thrombosis in a manner directly dependent on embedded cell expression of perlecan [47]. Such cell implants control the ordered healing of structures other than vessels as well, like the injured trachea [31] and now solid tumors [61]. Matrix-embedded endothelial cell implants in disease models can provide unique insights not easily obtainable by delivery of isolated factors.

Classical views of endothelial cells in cancer: role in tumor angiogenesis.

"The presence of a tumor-angiogenesis factor suggests a transfer of information from tumor cells to capillary endothelial cells. The relationship between tumor cells and endothelial cells may be interdependent." Folkman et al, *J. Exp. Med.*, 1971 [62].

Vascularization is essential for the development of physiologic and pathologic tissues. The *calor, tumor*, and *rubor* of inflammation arise from vascularization, and modern schemata of cancer biology must include vessels for continued growth and eventual metastasis [63-64]. The tumor angiogenesis paradigm as initially described by Folkman [63, 65] posited that since growing tumors need a dedicated blood supply for perfusion support, interruption of the blood supply should interrupt tumor growth. Normally a balance between pro-angiogenic and anti-angiogenic factors maintains vessel homeostasis, with vascular network expansion balanced by pruning. Tumor vessels' unchecked expansion is likely driven by the incorporation of endothelial cells derived from existing local vessels and perhaps circulating mature or progenitor endothelial cells as well [66]. Without this supporting vascular network tumors are unable to grow to more than ~1 mm^3 in volume, remaining small, dormant and relatively non-aggressive [63]. Once a tumor does achieve an "angiogenic switch", new vessels are recruited that first increase tumor microvascular density and later increase tumor growth and invasiveness.

Jain and colleagues extended the concepts surrounding perfusion mediated effects of tumor vessels by realizing that these vessels, comprised mainly of endothelial cells, possess abnormal architecture due to an imbalance of pro- and anti-angiogenic factors [67]. This architectural dysregulation includes heightened permeability, which contributes to intratumoral hypoxia and acidosis, and elevated interstitial pressure, which can facilitate the outward spread of cancers and impede soluble molecule flux into the tumor. Hypoxia can then directly contribute to tumor aggressiveness [68]. Intriguingly, they suggest that "normalization" of the tumor vasculature by doses of anti-angiogenesis agents insufficient to destroy the vasculature instead restores the balance of pro- and anti-angiogenic factors and partially explains the (modest) successes of such therapies. Other tumor endothelial phenotypic abnormalities include an "activated" integrin expression pattern [69], dysregulated leukocyte adhesion [70], abnormal responses to oxidative stress [71], and abnormal mechanosensing [72]. These same derangements have been characterized in dysfunctional endothelial cell phenotypes in vascular disease [17, 73].

Most of the explosive research in tumor angiogenesis concentrated on whole vessels: how they are recruited [63, 65] and structurally distorted [67] to promote tumor growth. Some have proposed a more direct role for the endothelial cells themselves in cancer regulation, including contact-dependent and -independent regulation [74-75]. Yet the homology to vascular repair has not been fully recognized and interest in such has receded in part. Moreover, the endothelial cell has been rarely [74-76] and inconsistently mentioned as part of the population of stromal cells such as fibroblasts

[77] and myeloid cells [78] that are increasingly recognized as essential elements of tumor biology. As "angiocrine" paradigms for stromal regulatory endothelial cells have begun to appear [79] it is worth considering how endothelial cell-derived paracrine regulatory models in the biology of vascular homeostasis and repair [17] can contribute to cancer sciences [61, 80].

The impact of anti-angiogenic therapy on cancer is revealing. Drugs designed to limit tumor vascularization can effectively shrinking tumors but have modest effects on patient survival [81]. Some have even proposed that specific modes of anti-angiogenic therapy that target VEGF might accelerate tumor invasion and metastasis while shrinking primary tumors [82-83]. Thus, there remain elusive details about the crosstalk between cancer cells and endothelial cells, and the effects of endothelial cell state – e.g. quiescent or dysfunctional – on such processes must be taken into consideration.

Tumor stroma: local regulators of the cancer cell microenvironment.

Solid tumors contain, in addition to cancer cells, stromal cells and paracrine crosstalk between cancer cells and cells of the microenvironment can regulate tumor proliferation, local invasion, and distant metastasis [84]. Two well-characterized cell types that contribute to cancer aggressiveness are fibroblasts and macrophages.

"Carcinoma-associated fibroblasts" (CAFs) are the predominant non-malignant cell type in most epithelial tumors. CAFs differ from normal tissue fibroblasts in that they are often contractile (myofibroblasts) and secrete matrix-metalloproteinases, collagenases,

extracellular matrix components, and a wide range of growth factors (HGF, IGF, VEGF, FGF, Wnt) and other factors (IL-6, SDF-1) [77]. Together these secretions directly support carcinoma cells and recruit blood vessels and other stromal cells to tumors. Interestingly, tumor-associated endothelial cells can also contribute to the CAF pool, sometimes in great numbers, by a process of transdifferentiation [85].

The immune system is similarly co-opted and locally modified by tumors. Immune cells may initially serve as sentinels, but can ultimately be used by cancer cells to circumvent immune recognition and attack [86]. Tumor-associated macrophages (TAMs), for example, block cytotoxic T cell-mediated actions via IL-10 secretion, generate free radicals, which can damage DNA, increasing the number of oncogenic mutations of cancers, and modulate NF-κB signaling [86]. Additionally, TAMs can recruit blood vessels, remodel the extracellular matrix to facilitate invasion and metastasis, and regulate local inflammation [78]. Conscripted regulatory T cells can aid cancer aggressiveness by attenuating the overall immune response to cancers [87]. Circulating leukocytes regulate and are regulated by endothelial cells [88], in a context-dependent manner in tumors and large vessels. Aberrant leukocyte homing, adhesion to, and passage through disarrayed tumor-associated endothelium is emerging as another element in this area [70].

Stromal cells of the cancer microenvironment are potentially attractive therapeutic targets. They are genetically stable, unlike transformed cancer cells, and offer a greater range of specific targets, with the potential for lower toxicity and intervention at multiple

and shared events in cancer evolution [89-90]. Furthermore, there may be means to utilize endogenous stroma to inhibit, rather than support, cancer aggression [91-92]. Strategies toward this goal could include both the imposition of pharmacologic or biophysical cues to normalize the tumor microenvironment or the placement of healthy regulatory cells within or adjacent to the deranged tumor milieu [61]. Along this line, it is intriguing that the cholesterol-lowering drugs that inhibit HMG-CoA reductase ("statin" drugs) and non-steroidal anti-inflammatory drugs (NSAIDs) both improve vascular integrity and endothelial health [93-94] and are associated with improved cancer prognoses [95].

Emerging "angiocrine" paradigm: dysfunctional tumor-associated endothelial cells.

Butler et al recently proposed a model [79] which combines the cancer-stroma interaction and angiogenesis paradigms. They proposed that endothelial cells are recruited to tumors to provide vascular paracrine, or "angiocrine," support for tumor growth and spread. This model has been used to identify, for example, that the endothelial cell EphA2 receptor negatively regulates the secretion of a cancer proliferation- and migration-stimulatory angiocrine factor Slit2 [80]. These notions are consistent with the increasingly appreciated roles of inflammation and stromal regulatory elements present in the tumor milieu [96], which could in combination cause endothelial cell dysfunction and, hence, endothelial cell support for diverse disease processes.

We posit that the disease-dominant angiocrine paradigm captures only part of the endothelial paracrine regulatory capabilities in cancer and that state-dependence might deepen this perspective (Fig. 1.1). We have mentioned two activated endothelial states ("physiologically activated" versus "dysfunctionally activated") that may form the ends of a regulatory spectrum, but there may be more relevant states in between, given the remarkably plastic nature of the endothelium [97-98]. Precisely as in atherosclerotic vascular disease, where reparative endothelial cells inhibit disease processes like inflammation, hyperplasia, or thrombosis, and dysfunctional endothelial cells stimulate the same, endothelial cells may regulate cancer cell pathophysiology in an analogous state-dependent manner. Reparative endothelial cells should suppress cancer cell malignant properties like proliferation or growth, invasion or metastasis, and dysfunctional endothelial cells stimulate the same. Regulatory factors already identified in vascular disease and repair may also contribute to cancer biology. Although advanced cancers must eventually recruit and corrupt the cells in their microenvironment, it may be possible to pharmacologically reverse the phenotype of endogenous tumor endothelial cells (from dysfunctional to quiescent) and regain control over the tumor milieu.

As in vascular disease, the subendothelial matrix and supporting membranes should also contribute to tumor biology by regulating endothelial state and thereby affect adjacent cancer cells within a tumor. Control over cell-matrix interactions may help to ensure that endothelial cells placed within or adjacent to the tumor milieu remain quiescent to overcome or resist the disruptive stimuli - regaining control of the tumor

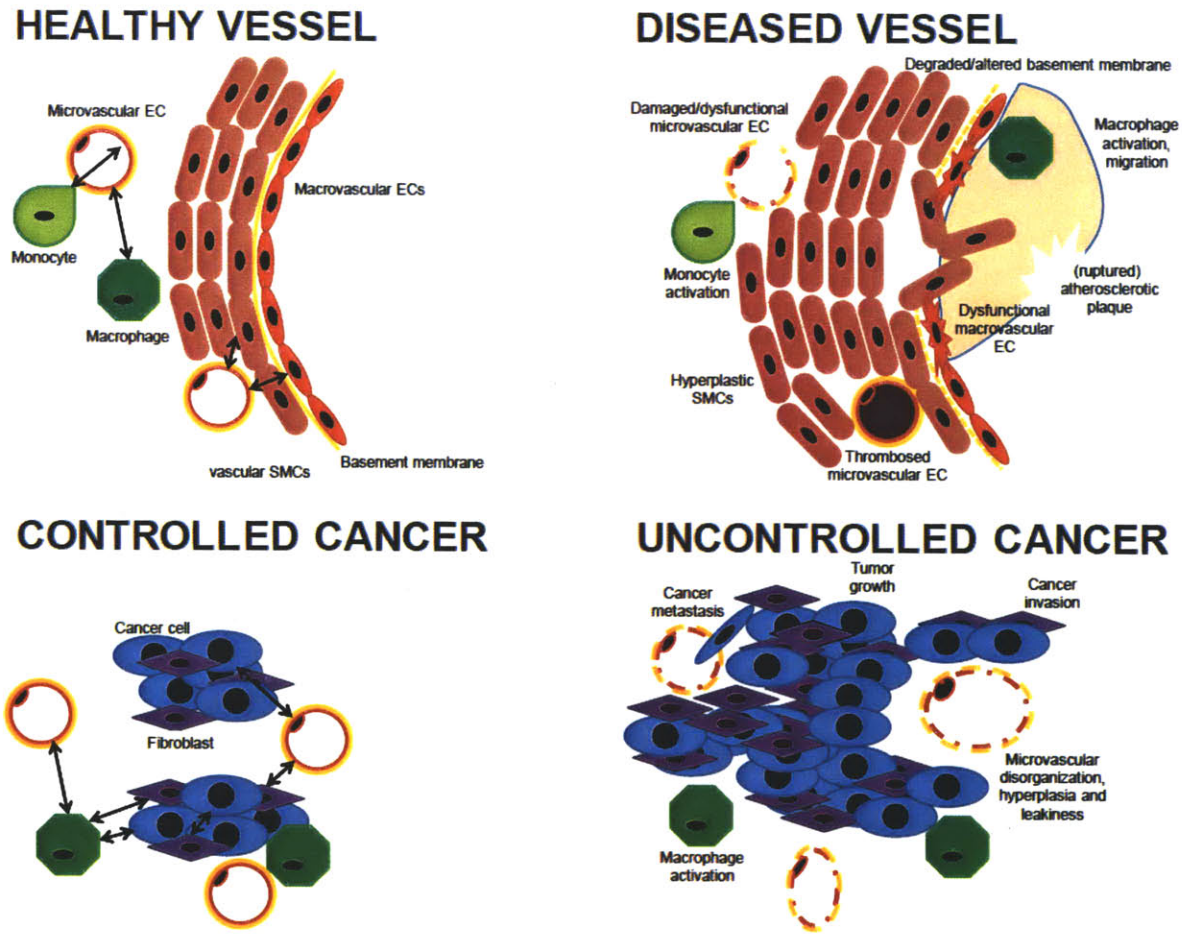

Figure 1.1. Similarities between endothelial phenotypes in vascular disease and cancer.

Quiescent endothelium is structurally normal and may be physiologically activated to inhibit diverse diseases processes such as vascular smooth muscle cell hyperplasia, occlusive vascular thrombosis, leukocyte attraction and migration, and inflammation. However, pathologically hyperactivated "dysfunctional" endothelium is structurally disorganized and promotes vascular disease processes. This bipotential regulatory status of the endothelium may also be important in cancer regulation, with quiescent endothelial cells serving as cancer-inhibitory regulators and dysfunctional endothelial cells as cancer-stimulatory regulators.

microenvironment to promote homeostasis. We recently showed that secretions from healthy, reparative endothelial cells suppress cancer cell proliferation, invasiveness, and inflammatory signaling *in vitro*, and tumor growth and experimental metastasis *in vivo* [61]. These experiments utilized the intact endothelial cell secretome, as opposed to constituent isolated factors, as a cancer-regulatory unit. The cell biologic approach adheres to the philosophy that paracrine regulation by intact cells is more than the sum of the actions of the individual factors. Combinations of individual factors can elicit even qualitatively different responses from target cells depending on dose and presence of cofactors. Prior work from our laboratory identified context-dependent roles for endothelial heparan sulfate proteoglycans in inhibiting vascular smooth muscle cell proliferation and showed how other factors emitted by endothelial cells either augment or reverse such inhibition [28]. Our use of matrix-embedded endothelial cell implants to control the behavior of solid tumors in animals was another example in which prior work in vascular repair presaged experiments in cancer. Quiescent matrix-embedded endothelial cell implants reduced tumor growth and normalized tumor structure [61], just as such implants help to guide repair after vascular [58, 60, 99] and epithelial injury [31].

Although we have consistently found that it is the synergistic action, with optimal dose and kinetics, of endothelial cell-secreted factors that most efficiently guides repair [28], manipulation of specific endothelial cell-secreted products can be useful to elucidate partial mechanisms of endothelial-derived regulation. We previously showed that silencing endothelial expression of perlecan, the predominant endothelial-secreted heparan sulfate proteoglycan, abrogated the ability to inhibit occlusive vascular

thrombosis, but not intimal hyperplasia, after vascular injury [47]. One could then surmise that perlecan expression is critical for maintenance of the disease-inhibitory endothelial cell phenotype. We therefore hypothesized that endothelial perlecan expression may play a role in endothelial anti-cancer effects. Indeed, that the knockdown of perlecan caused a transcriptional upregulation of IL-6 and eliminated the ability of endothelial cells to inhibit cancer cell invasion and metastasis [61] supports the general vascular-cancer paradigm homology and sharing of specific regulatory mechanisms, perhaps by similar sets of secreted regulators. Thus the phenotype of tumor-associated endothelium could be modified either by direct action of molecular mediators on the endothelial cells themselves or by modification of the subendothelial basement membrane.

The transfer of regulatory paradigms from vascular repair to cancer may be useful in identifying previously unrecognized processes in endothelial cell-cancer crosstalk. High-throughput gene expression studies have offered abundant data [100-102], but few tumor endothelial genes have been consistently identified as candidate paracrine regulators. Intriguingly, of the genes that have been identified many encode ECM structural or remodeling molecules [102], which could support the notion that particular modifications of the subendothelial membrane contribute to tumor progression by modification of the endothelial phenotype.

Summary: Paracrine context-dependent regulatory roles of endothelial cells in cancer.

> "Perhaps you will be kind enough to look on what I have said today as one more interim report on endothelium. Our knowledge is still far from being definitive, and I should expect to see the next ten years yield a rich harvest of new knowledge about the cells which stand between the blood and lymph streams and the cells of the tissue. I would expect to see exemplified the dicta that the introduction of a new technique is certain to be followed by new discoveries and that the pushing of a known technique to greater heights of technical achievement will produce new accretions of knowledge." Lord Florey [1].

We propose that the endothelial cell paracrine regulatory phenotype is global and plays an important role in all diseases, given the uniquely privileged anatomic position of microvascular ECs in all tissues. We further hypothesize that endothelial regulation of inflammatory signaling – inhibition by reparative endothelial cells and stimulation by dysfunctional endothelial cells – plays a particularly important role in this regulatory phenotype. The list of diseases impacted by endothelial anti- and pro-inflammatory regulation is immense, and includes atherosclerosis [103-104], hypertension [105], uremia [106], diabetes mellitus [107], and now perhaps cancer [61, 79-80].

As with the foundational work of Virchow, Florey and all of those who came before us, our views will certainly be refined with continued investigation. We have attempted to merge traditionally distinct fields of study into an updated report of the roles of the endothelium in health and disease. We hope that studies using matrix-embedded endothelial cells as convenient and controllable cellular implants will help define the extent and limits of cancer-endothelial cell crosstalk. Such work may also enable the design of pharmacologic therapies to reverse tumor endothelial phenotype from dysfunctional to reparative and guide the design of quiescent cellular implants that are able to resist the pressures present in the tumor milieu to effectively and permanently "heal" tumors.

CHAPTER 2: STROMAL ENDOTHELIAL CELLS DIRECTLY INFLUENCE CANCER PROGRESSION

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Abstract

Although the roles of endothelial cells in cancer have been primarily considered to be related to tumor perfusion, there is emerging appreciation of "angiocrine" regulation - adding stromal regulatory capabilities to the expanding list of endothelial functions in tumors. We posit that the state-dependent paracrine regulatory paradigms established in vascular disease and repair will be critical for a deep understanding of tumor biology, as endothelial cells regulate diverse physiologic and pathophysiologic processes in all vascularized tissues. We now outline the historical developments that led to the appreciation of the paracrine regulatory functions of endothelial cells, summarize classical views of blood vessels and stroma in cancer, and attempt to merge these ideas to include the stromal regulatory endothelial cell as a critical regulator of cancer. The notion of the endothelial cell as a biochemical regulator of cancer state in constant dynamic balance with its tumor could impact diagnosis, prognosis and treatment of cancer. Such concepts might well explain the mixed results from anti-angiogenic cancer therapeutics and how certain drugs that improve vascular health correlate with improved cancer prognosis.

Introduction

Tumor growth and metastasis depend critically on cellular and vascular elements. Indeed, Folkman seized on the vascular nature of tumors to propose that angiogenesis was rate-limiting for tumors and suggested anti-angiogenesis therapies for cancer treatment [65]. Tumor vessels were originally thought to control tumor growth through perfusion of metabolically active cancer cells [63]. Tumor growth and dissemination was envisioned to arise in part from an imbalance in pro-angiogenic and anti-angiogenic growth factors [63]. More recently, the leakiness of tumor blood vessels has been indicted as contributing directly to tumor growth and metastasis by increasing tumor interstitial pressure (e.g. facilitating efflux of cancer cells), and by creating foci of hypoxia, and acidosis [67]. Clinical trials of anti-angiogenesis cancer therapies, however, have shown mixed results, with initial reduction in tumor burden [108-109], but no significant extension of long term patient survival [81, 110] and even a potential increase in cancer invasion and metastasis [82-83].

The contemporary view of cancer envisions tumors as "ecosystems" [111-112] consisting not simply of proliferating cells alone but of diverse collections of recruited stromal cells that regulate cancer behavior [78, 86-87, 89-90, 113]. The endothelial cells (ECs) that line blood vessels are the first cells in contact with any blood-borne element and are especially prevalent in tumors [114]. ECs are also critical to the biology of normal tissues; tissue health is often synonymous with endothelial integrity [29, 31, 47, 97, 115]. This is especially true in the vascular system, where ECs promote homeostasis when quiescent by suppressing local hyperplasia, angiogenesis, and

inflammation, and enhance injury by stimulating these processes when they are diseased or "dysfunctional." We hypothesize that ECs serve a similar role in tumors. In this paradigm ECs, like other stromal cell types, regulate cancer cell behavior, promoting homeostasis when healthy and stimulating cancer when dysfunctional. In this paradigm ECs function not simply as static structural cells of perfusing vessels but as active stromal regulatory cells with privileged access to the deepest recesses of tumors. Subtle changes in EC phenotype could be then easily transmitted to the tumors with profound effects on cancer fate.

We now show that ECs can regulate diverse aspects of cancer cell function, including proliferation, invasiveness, and response to and elaboration of inflammatory mediators *in vitro*, as well as tumor growth and metastasis *in vivo*. Moreover, we demonstrate that altering the EC secretome can have a profound impact on these cancer-regulatory phenomena. These findings add to an emerging appreciation of potential EC cancer-regulatory effects that transcend the role these cells play as lining of a tumor-perfusing vascular network and offer new modes of cancer diagnosis, prognostication, and therapy.

Materials and Methods

Chemicals and Reagents

Antibodies to Ki67 were from Santa Cruz Biotechnology, to NF-κB p65, p-S6RP, p-STAT3, β-actin, and MMP2 from Cell Signaling Technology, to PCNA from Abcam, and to IL-6 from RnD Systems. HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology. Fluorescently-labeled secondary antibodies were from Invitrogen, rapamycin from Sigma, and DAPI and oligonucleotide PCR primers were from Invitrogen.

Cell culture

Primary human umbilical vein ECs (HUVECs, Invitrogen) were cultured in EGM-2 (Lonza) with an additional 3% FBS on gelatin-coated TCPS plates and used between passages 2-6. Cells were passaged by detachment with trypsin and split 1 to ~5. EC-conditioned media were generated from confluent HUVEC monolayers by 48 hours of culture in MCDB131 (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells and debris were removed by centrifugation (5 minutes, 500g) and media were aliquotted and stored at -80°C. Primary human lung fibroblasts (Lonza) were cultured in the same manner as ECs. A549 (lung carcinoma) and MDA-MB-231 (breast carcinoma) cells (ATCC) were cultured on TCPS dishes. Cells were cultured under standard conditions (37 °C, 5% CO_2)

Matrix-embedded EC (MEECs) were generated by culturing ECs within sterile Gelfoam compressed matrices (Pfizer, New York) [31]. Matrices were cut into

1.25x1x0.3 cm blocks and hydrated in EC growth medium at 37°C for 2-48 hours. ECs were suspended in medium, seeded onto hydrated blocks and allowed to attach for 1.5 hours, then the contralateral side seeded with an equal number of cells. After additional time for cell engraftment two blocks were added to 30 mL polypropylene tubes containing 6 mL of EGM-2. MEECs were cultured for up to 3 weeks, with media changed every 48-72 hours, under standard culture conditions. Samples from each lot were digested with collagenase (type I, Worthington Biochemicals) and cell seeding efficiency determined with a Z1 Coulter particle counter (Beckman Coulter; Fullerton, CA). Cell viability was assessed by trypan blue exclusion.

Knockdown of perlecan in ECs

pLKO.1 plasmids containing shRNA against perlecan, and as a control pLKO.1 without shRNA, (Open Biosystems, Huntsville, AL) were grown in transformed bacteria, purified (PureLink HiPure system, Invitrogen), and used to transfect HEK-293T packaging cells using Lipofectamine 2000 (Invitrogen). Packaging, envelope, and Rev plasmids were co-transfected simultaneously as described elsewhere [116]. Viral particles were collected for 48 hours and transferred, along with 10 μg/mL hexadimethrine bromide, to subconfluent EC monolayers. Puromycin (1 μg/ml) was used for selection of stably transduced ECs.

In vitro tube forming assay

15,000 ECs were seeded in each well of 96-well plate coated with 50 μL of Matrigel (BD Biosciences). After 16-20 hours, tube formation was imaged by phase contrast microscopy. ImageJ was used to quantify tube length, using 4 wells per condition.

Gene expression analysis

Total RNA was purified (RNEasy Mini Plus, Qiagen) and cDNA was synthesized (TaqMan reverse transcription reagents, Applied Biosystems) using 1 μg of RNA. Real-time PCR analysis was performed with an Opticon Real-Time PCR Machine (MJ Research) using SYBR Green PCR Master Mix (Applied Biosystems) and appropriate primers. Gene expression was quantified using the ΔΔCt method, with GAPDH as a housekeeping gene. Primer sequences are listed below.

	forward 5' - 3'	reverse 5' - 3'
GAPDH	ACAGTCAGCCGCATCTTCTT	TGGAAGATGGTGATGGGATT
MMP2	AACGGACAAAGAGTTGGCAG	GTAGTTGGCCACATCTGGGT
MT1-MMP	TGATAAACCCAAAAACCCCA	CCTTCCTCTCGTAGGCAGTG
TIMP1	GGAATGCACAGTGTTTCCCT	GAAGCCCTTTTCAGAGCCTT
TIMP2	TGATCCACACACGTTGGTCT	TTTGAGTTGCTTGCAGGATG
Perlecan	ATTCAGGGGAGTACGTGTGC	TAAGCTGCCTCCACGCTTAT
IL-6	CACAAGCGCCTTCGGTCCAGTT	TCTGCCAGTGCCTCTTTGCTGC

Protein expression and Western blotting

Whole cell extracts were harvested with buffer containing 0.5% Triton X-100, 0.1% SDS, protease inhibitor cocktail (Roche), 2 mM sodium orthovanadate, 50 mM sodium fluoride, and 4 mM PMSF. Protein samples were separated on glycine-SDS gels, transferred to nitrocellulose membranes, immunoblotted with the appropriate primary

and HRP-conjugated secondary antibodies, treated with a chemiluminescent peroxidase substrate (SuperSignal West Femto, Pierce). Luminescence was measured by a FluorChem luminometer (Alpha Innotech; CA) and analyzed using ImageJ. A cytokine antibody array (RayBiotech; GA) was used following the manufacturer's instructions for assessment of cell biosecretions. Array luminescence was imaged using a FluorChem luminometer (Alpha Innotech; CA) and quantified using ImageJ. IL-6 present in cell culture supernatants was assayed with an EIA kit (RnD Systems, Minneapolis, MN) according to the manufacturer's instructions.

Gelatin Zymography

Samples were run on 10% polyacrylamide gels containing 0.1% gelatin (Invitrogen) under non-denaturing conditions. Recombinant human MMP2 (RnD Systems, Minneapolis, MN) was either used directly or after activation with 1 mM APMA (Sigma) and used as a control. Separated proteins were renatured with Triton X-100 (Invitrogen) and then allowed to digest the gelatin overnight at 37°C. Areas of gelatin digestion were detected by using a non-specific protein stain (Invitrogen) and the gels were scanned and analyzed using ImageJ.

Chemoinvasion/chemomigration assay

Chemoinvasion kits (BioCoat, Becton Dickinson) were used according to the manufacturer's instructions. Invaded or migrated cells adherent to the bottom of the inserts were fixed, stained with DAPI (1 μg/mL, 30 minutes) and imaged by epifluorescence microscopy. Images were analyzed by visual inspection and cytometric

quantification of 4 random 20X fields at the microscope or by using the "particle counter" feature of ImageJ for the central 10X field. Data are expressed as an invasion index [117], the average number of invaded cells and the average number of migrated cells of a given condition, normalized to the control condition with at least 3 wells used per condition.

Xenograft tumor and tail vein metastasis in vivo models

All *in vivo* experiments were approved by the Massachusetts Institute of Technology Committee on Animal Care and comply with NIH guidelines. Female nude mice, aged ~6 weeks, were purchased from Charles River Labs (Wilmington, MA) and housed in sterile cages with sterile bedding, food (ad libitum), and water. After allowing at least 4 days to adjust to the animal facility, mice were used in one of the following models.

For the primary xenograft tumor model 5E6 human A549 lung carcinoma cells were injected (suspended in 100 μL HBSS after harvesting and rinsing 2X with HBSS to remove serum and trypsin) subcutaneously on the dorsal surface. After allowing 12 days for tumor engraftment, either acellular Gelfoam (control) or MEECs (~1E6 cells per animal) were twice with HBSS and implanted adjacent to the tumors; the surgical site was sealed with tissue clips. At the end of the experiment, animals were sacrificed by CO_2 inhalation. All surgeries were performed under anesthesia with 2% isoflurane delivered via nose cone and 0.1 mg/kg buprenorphine administered perioperatively. Tumors were measured 1-2 times/week with Vernier calipers, using two measurements to estimate the volume, assuming a prolate spheroid geometry.

Tail vein metastasis experiments followed established protocols [118]. Briefly, exponentially-growing A549 lung carcinoma cells were trypsinized, washed twice in PBS, resuspended at a density of 5E6 cells/mL, and injected into the tail vein of mice under 2% isoflurane anesthesia. 22 days after cell injection, the animals were sacrificed (CO_2 inhalation) and the lungs explanted, fixed, weighed, and cryosectioned. To generate a "metastasis staining index" three 20X fields per lung section, four sections per animal, were examined for the presence of exogenously seeded A549 cells. The average fluorescent intensity was determined from of eight 80x80 pixel boxes in each field of a given image. The early time point was selected to study invasion and colonization of the lungs – more in line with *in vitro* work – rather than subsequent secondary tumor growth.

Immunofluorescent staining and epifluorescence microscopy

Cells in chamber slides were washed, fixed (10 minutes, 4% paraformaldehyde, room temperature), permeabilized with 0.25% Triton X-100, and incubated with primary antibodies overnight at 4°C. Fluorescently-labeled secondary antibodies were added, along with 1 μg/mL DAPI, for two hours at room temperature in the dark. Cells were then washed, coverslipped (ProLong Gold antifade medium, Invitrogen), and imaged using an epifluorescence microscope (Leica). Images were analyzed using ImageJ. Excised primary tumors and lungs were flash frozen in liquid nitrogen-cooled isopentane. 10-μm frozen sections were cut using a cryotome, fixed for 10 minutes with acetone at -20°C, blocked with serum/BSA/PBS for 45 minutes at room temperature,

and stained with appropriate primary and fluorescence-conjugated secondary antibodies as described for cells.

Statistical Analyses

All experiments were performed at least thrice for validation and each time in triplicate, at minimum; results are expressed as mean$\pm$SEM. Comparison of two groups was performed using a student's t-test. Comparison of multiple ($\geq$ 2) groups was performed using ANOVA followed by t-tests. $p < 0.05$ was taken as statistically significant.

Results

Secretions from quiescent ECs reduce cancer cell proliferation and invasiveness.

We assayed how culture in EC-conditioned media affects cancer cell proliferation. Media conditioned by confluent ECs reduced growth of MDA-MB-231 breast and A549 lung carcinoma cells by ~40% ($p < 0.001$ for both, Fig. 2.1, Fig. 2.2A). The reduction in cell number correlated with a $35\pm12\%$ ($p < 0.05$) and $44\pm9\%$ ($p < 0.05$) decrease in PCNA expression (Fig. 2.2B) and with a $23\pm5\%$ ($p < 0.05$) and $45\pm25\%$ ($p < 0.05$) reduction in the fraction of cancer cells with Ki-67 positive nuclei (Fig. 2.2C).

Cancer cell invasiveness is a key trait in determining the aggressiveness and metastatic potential of tumors. Migration and invasion were measured in a dual chamber culture system. Migration was measured by passage of cancer cells through 8 μm porous membrane inserts into a chemokine-filled chamber, and invasion by cancer cells passing through pores coated with Matrigel [117]. Four days of culture in EC–conditioned media significantly reduced *in vitro* invasiveness of both cancer cell lines (Fig. 2.3A). Migration was unchanged in both cancer cell types (126 ± 27 vs. 133 ± 42 cells/field for MDA-MB-231, 336 ± 28 vs. 331 ± 85 for A549), and all of the effect seen in the invasion index was from changes in invasion (33 ± 6 vs. 24 ± 7 for MDA-MB-231, 50 ± 3 vs. 25 ± 12 for A549). Intriguingly, gene expression associated with reduced invasiveness was different in the two cancer lines. Inhibition of invasion in MDA-MB-231 cells was accompanied by a 4.2 ± 0.9 fold reduction ($p < 0.01$) in ECM pro-remodeling enzyme MMP2 expression, whereas the effects on A549 cells were associated with an increase in MMP inhibitors, including a 1.7 ± 0.4 fold increase ($p < 0.05$) in expression of

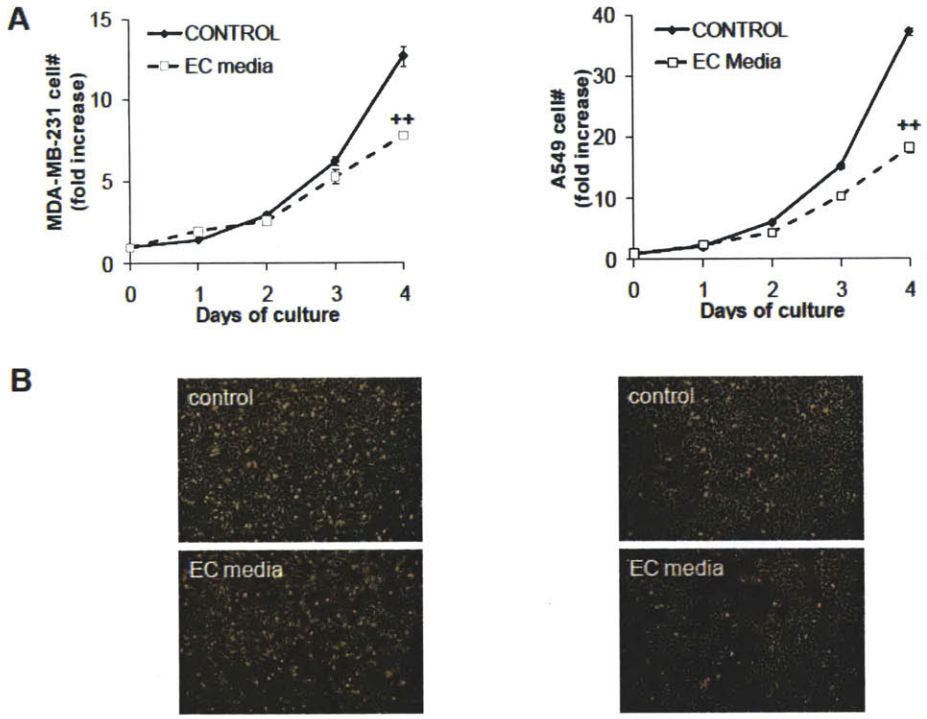

Figure 2.1. Long-term culture of cancer cells in endothelial cell-conditioned media slows cell growth.

(A) Four days after seeding, MDA-MB-231 breast and A549 lung cancer cell number was statistically significantly lower when cultured in media conditioned by quiescent EC compared to cancer cells in control media. Each point represents duplicate measures of at least three different wells. ($^{++}$ = $p < 0.001$) (B) Although cancer cell density and proliferation was affected cell morphology remained unaffected by EC-conditioned media as visualized by phase contrast microscopy.

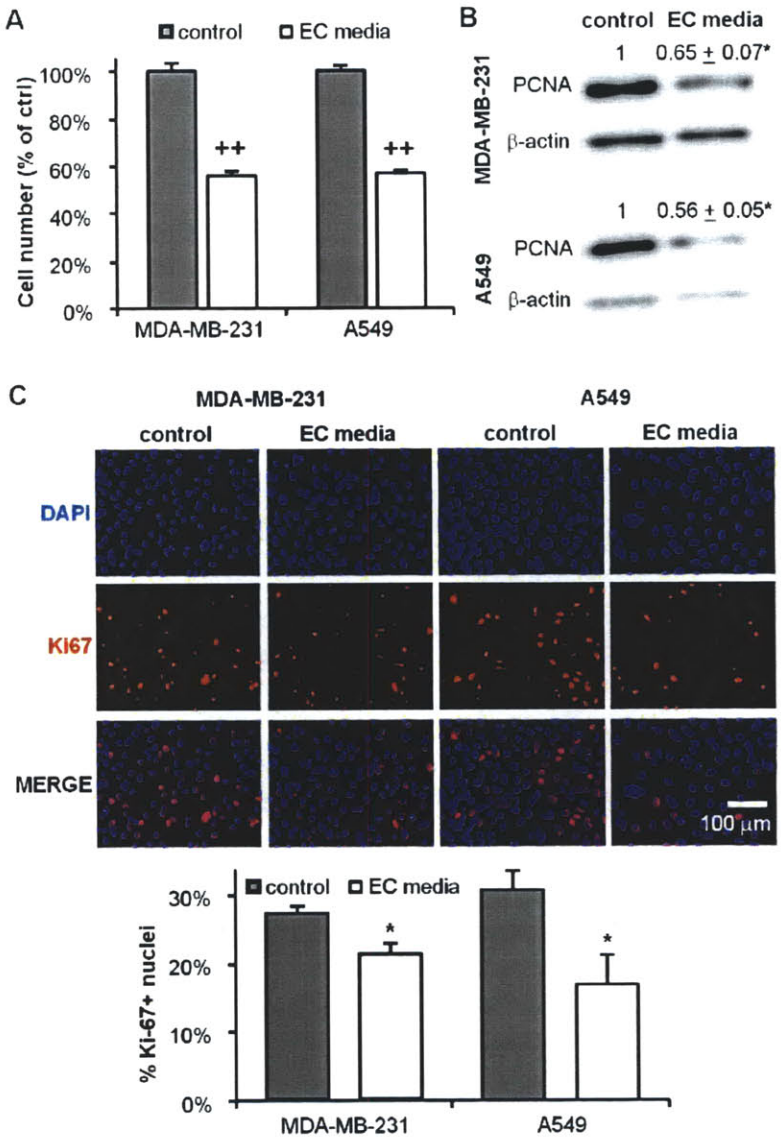

Figure 2.2 Quiescent endothelial cells (ECs) secrete factors that suppress cancer cell proliferation.

(A) Growth of MDA-MB-231 breast and A549 lung carcinoma cells for 4 days in unconditioned (control) or EC-conditioned media. (B) Expression of proliferating cell nuclear antigen (PCNA) protein in cancer cells by Western blot. (C) Ki-67 nuclear expression via immunofluorescence staining in the same groups. * $p < 0.05$ versus control by t-test. Error bars show SEM.

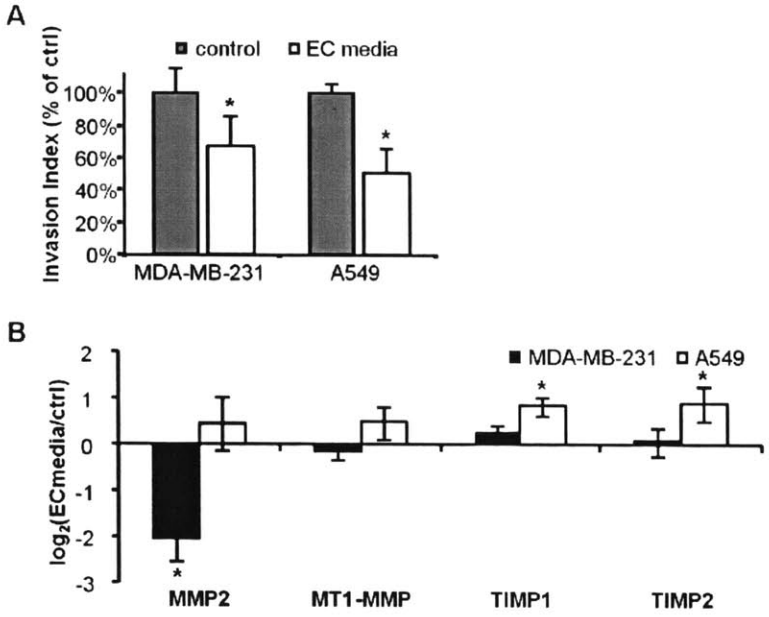

Figure 2.3. Quiescent ECs secrete factors that suppress cancer cell invasiveness.

(A) Invasiveness of MDA-MB-231 breast and A549 lung carcinoma cells after 4 days of culture in unconditioned (control) or EC-conditioned media. (B) Selected matrix-regulating gene expression (qRT-PCR) of both lines under the same treatment conditions. * $p < 0.05$ versus control by t-test. Error bars show SEM.

TIMP1 and a 1.8±0.7 fold ($p < 0.05$) increase in TIMP2 (Fig. 2.3B). However, even the activities of these individual proteins does not account for the entire observed effect. Indeed, although MMP2 can enhance cancer cell invasiveness [119], and ECs secrete MMP2 [120] and deposit this enzyme on cancer cells, the EC secretome successfully inhibited cancer invasiveness despite the presence of deposited MMP2 or exogenously administered activated MMP2 (Fig. 2.4).

Conditioned media from confluent fibroblasts served as a control and had no effect on cancer cell proliferation or invasiveness (Fig. 2.5). These findings suggest a specific regulatory role for quiescent ECs in promoting homeostasis by suppression of both aberrant cancer cell proliferation and invasiveness.

Multiple tumorigenic pathways in cancer cells are affected by culture in EC secretions.

We examined a subset of signaling pathways that contribute to cancer cell biology, including those signals that govern cell growth and proliferation, like the mTOR pathway [121] and pro-metastatic NF-κB and STAT3 pathways critical for inflammatory signaling [122]. Each of these signals is regulated by ECs in vascular repair and disease [123-124]. Four days of culture in EC-conditioned media reduced significantly phosphorylation of S6 ribosomal protein and STAT3β, and decreased the total NF-κB p65 in both cell lines similarly ($p<0.05$, Fig. 2.6A). Phosphorylation of S6RP fell 76±9% for MDA-MB-231 and 64±6% for A549, and STAT3β phosphorylation decreased by

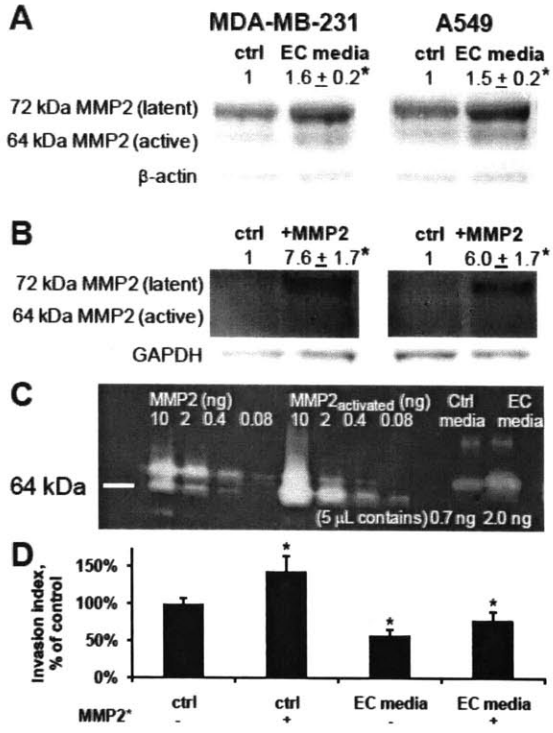

Figure 2.4. Although the secretome of ECs contains a large amount of latent MMP2, it inhibits significantly cancer cell invasiveness.

(A) Western blots of whole cell lysates of MDA-MB-231 and A549 cells cultured for 4 days in endothelial cell (EC) conditioned media show an approximately 50% increase in total (latent and active) MMP2 relative to cells cultured in unconditioned media. (B) This effect can be recapitulated by culturing the cells in the presence of recombinant human MMP2. (C) Zymography confirms that EC-conditioned media contains ~ 300 ng/mL more latent MMP2 than unconditioned media. Standard curves of latent (left) and activated (right) enzyme were used to estimate this value. (D) recombinant MMP2 alone enhances the invasiveness of A549 cells (44±22%, $p<0.05$) unless these cells had been cultured previously for 4 days in EC media. EC media inhibits invasiveness (41±16%, $p<0.05$) and can even overcome the EC deposition of MMP2 on cancer cells. Invasiveness of cancer cells after exposure to MMP2 and EC conditioned media was suppressed statistically identically to the manner in which media alone acted on cancer cells (41+15% vs. 22±13% reduction of invasiveness, p=N.S.).

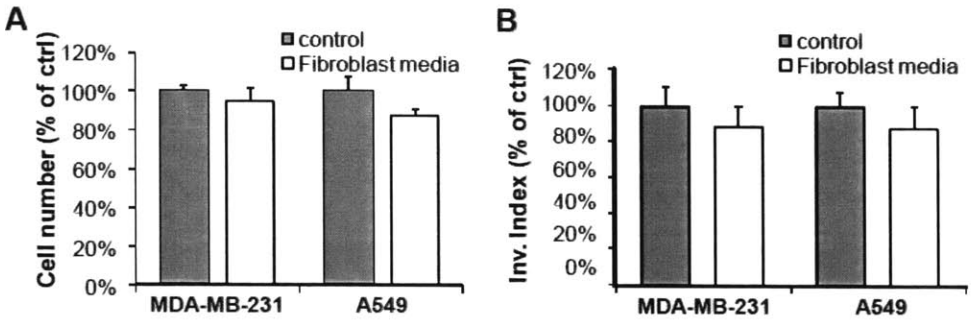

Figure 2.5. Media conditioned by normal fibroblasts have no effect on cancer cell proliferation or invasiveness.

(A) Effects of media conditioned by normal human lung fibroblasts (NHLFs) on MDA-MB-231 breast and A549 lung carcinoma cell numbers after 4 days in culture. (B) Effects of media conditioned by NHLFs on the invasiveness of MDA-MB-231 and A549 cells after 4 days in culture.

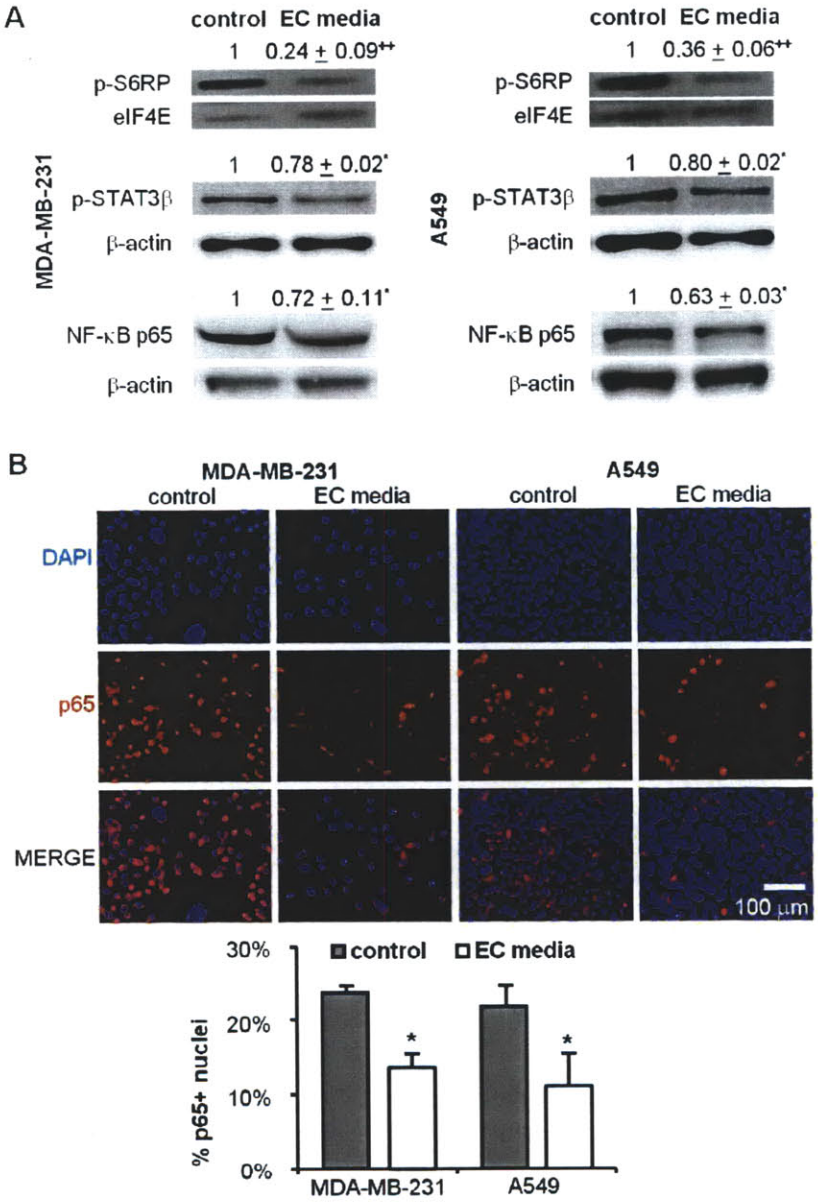

Figure 2.6. Signaling through pro-tumorigenic and pro-inflammatory pathways is attenuated when cancer cells are cultured with media conditioned by quiescent ECs.

(A) Phosphorylation of S6RP and STAT3 and total expression of NF-κB p65 in MDA-MB-231 and A549 cells after 4 days of culture in EC-conditioned media, with b-actin as a laoding control. (B) Nuclear localization of NF-κB p65 by immunofluorescence staining of both cell types. * $p < 0.05$ versus control by t-test. Error bars show SEM.

22±2% for MDA-MB-231 and 20±2% for A549. Total NF-κB was decreased by 28±11% for MDA-MB-231 and 37±3% for A549 cells relative to culture in control media. Additionally, the nuclear localization of NF-κB p65 was reduced by 41±7% for MDA-MB-231 and 50±15% for A549 after culture in EC-conditioned media ($p<0.05$, Fig. 2.6B).

Signaling changes in one pathway might cause lateral signaling changes in other pathways. Since we observed the largest reduction in expression of p-S6RP after culture in EC media, we examined whether inhibition of mTOR signaling alone could reproduce our effects. Rapamycin (0.13 μg/mL) completely inhibited phosphorylation of S6RP but only slightly reduced the number of cancer cells after 4 days ($p < 0.05$, Fig 2.7A) and had no significant effect on STAT3β phosphorylation or the total amounts of NF-κB p65 (Fig. 2.7B). These data suggest that the changes in cancer cell proliferation and invasion are effected by modulating multiple regulatory pathways, and possibly via the actions of multiple EC-secreted molecules.

Perlecan knockdown increases EC inflammatory secretions and eliminates EC ability to suppress cancer invasiveness

Perlecan, the major extracellular heparan sulfate proteoglycan expressed by ECs, is a complex regulator of vascular biology and tumor angiogenesis [47-48]. ECs were stably transduced with a lentiviral plasmid containing shRNA targeting perlecan (Fig. 2.8A). Such ECs (EC_{shPerl}) expressed 55±11% less perlecan mRNA than control ECs (qRT-PCR, $p < 0.01$, Fig. 2.8B). Perlecan silencing did not change EC morphology (Fig.

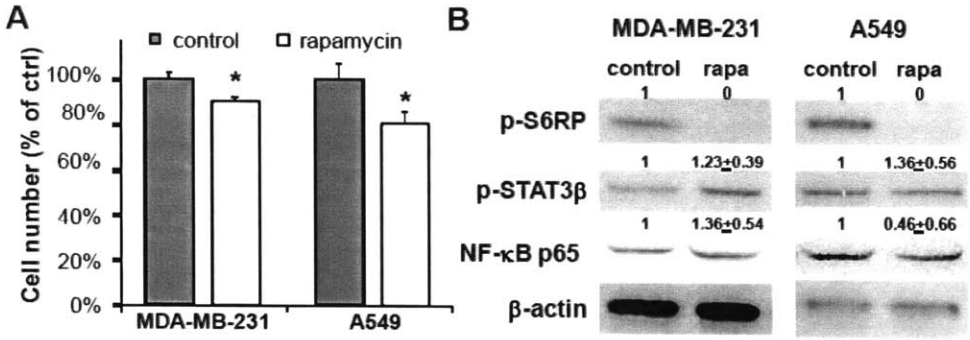

Figure 2.7. Inhibition of one signaling pathway in cancer cells cannot recapitulate EC-mediated regulation of cancer cells.

Rapamycin reduced MDA-MD-231 and A549 proliferation 96 hours after exposure by 10% and 17% (A) in concert with almost complete elimination of S6RP phosphorylation but, in contrast to culture in EC-conditioned media, without affecting the phosphorylation of STAT3 or the total levels of NF-kB p65 (B).

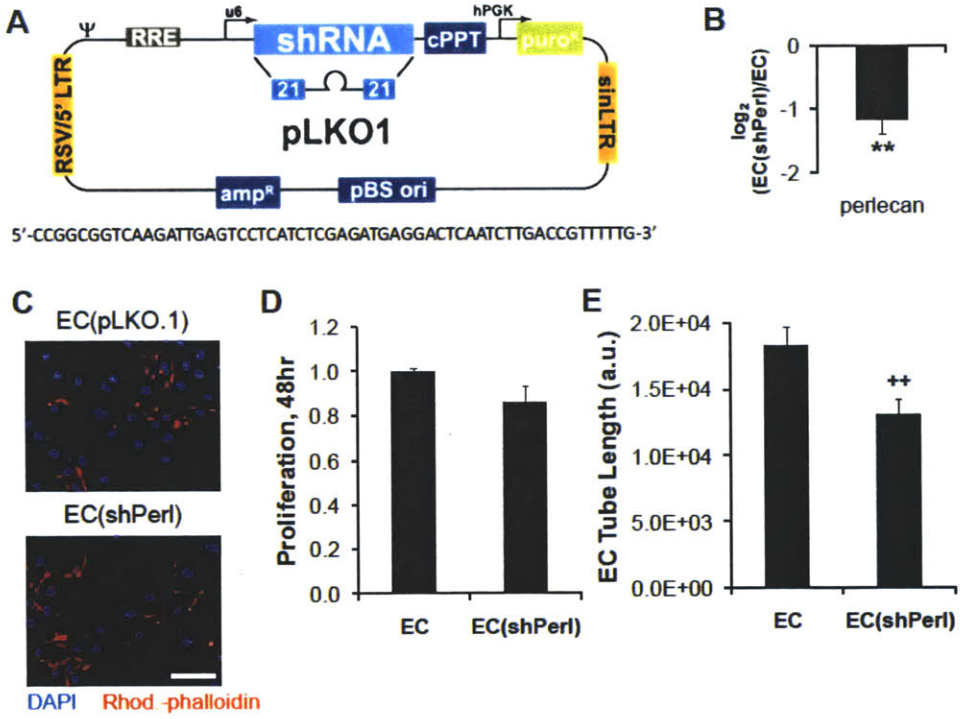

Figure 2.8. Description of perlecan silencing on EC phenotype.

(A) Schematic diagram and the shRNA coding sequence of the lentiviral plasmid used for delivery of shRNA targeting perlecan mRNA into ECs. (B) Perlecan mRNA expression in EC is reduced by 55±11% ($p < 0.01$) after transduction with a lentiviral plasmid containing shRNA against perlecan, without much affect on EC morphology (C) and without a statistically significant reduction in EC proliferation, (D) and with only modest reduction (28±1%, $p < 0.001$) of endothelial tube formation (E).

2.8C) or growth kinetics (Fig. 2.8D) significantly, yet it did reduce EC tube-forming capabilities on Matrigel ($p < 0.001$, Fig. S5E). Interestingly, media conditioned by EC_{shPerl} had a slightly increased inhibitory effect on cancer cell proliferation compared to media conditioned by control-transduced ECs but could no longer suppress invasiveness of either MDA-MB-231 (Fig. 2.9A, p = N.S.) or A549 cells (Fig. 2.9B, p = N.S.).

Since perlecan can bind many growth factors and cytokines [48], we assayed the effects of silencing this proteoglycan on EC cytokine release. Perlecan-silenced ECs (EC_{shPerl}) released 4.5 times more interleukin-6 (IL-6) into medium compared with EC transduced with a control plasmid ($p < 0.001$, Fig. 2.10A); release of IL-8, GRO, and GRO-α also increased but more modestly ($p < 0.001$, Fig. 2.10A). To determine if the increased IL-6 release from EC_{shPerl} was responsible for the differential effects on cancer phenotype, EC_{shPerl}-conditioned media was pre-incubated with 50 μg/mL IL-6 neutralizing antibody or isotype-control IgG antibody before use in cancer cell cultures. IL-6 neutralization had no effect on the inhibition of cancer cell proliferation by EC_{shPerl}, but completely restored the ability of media conditioned by EC_{shPerl} to inhibit cancer cell invasiveness (Fig. 2.10B,C). Recombinant IL-6 (10 ng/mL) completely abrogated EC suppression of cancer cell invasiveness (Fig. 2.11A). These findings are consistent with the established role of IL-6 in promoting cancer cell invasive/metastatic behavior [125-126] and imply that the increased IL-6 secretion with perlecan silencing induced differential effects of EC secreted factors on cancer cell invasiveness.

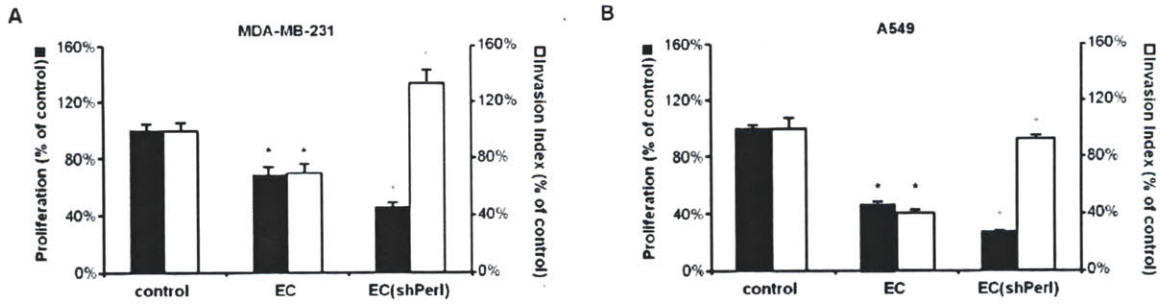

Figure 2.9. EC perlecan expression is required for EC-mediated suppression of cancer cell invasiveness.

(A and B) Proliferation (black bars) and invasiveness (white bars) of MDA-MB-231 (A) and A549 (B) cells after 4 days of culture in unconditioned (control) media, media conditioned by unmodified ECs, and media conditioned by perlecan-silenced ECs (ECshPerl). * $p < 0.05$ (black versus control, gray versus EC) by t-test. Error bars show SEM.

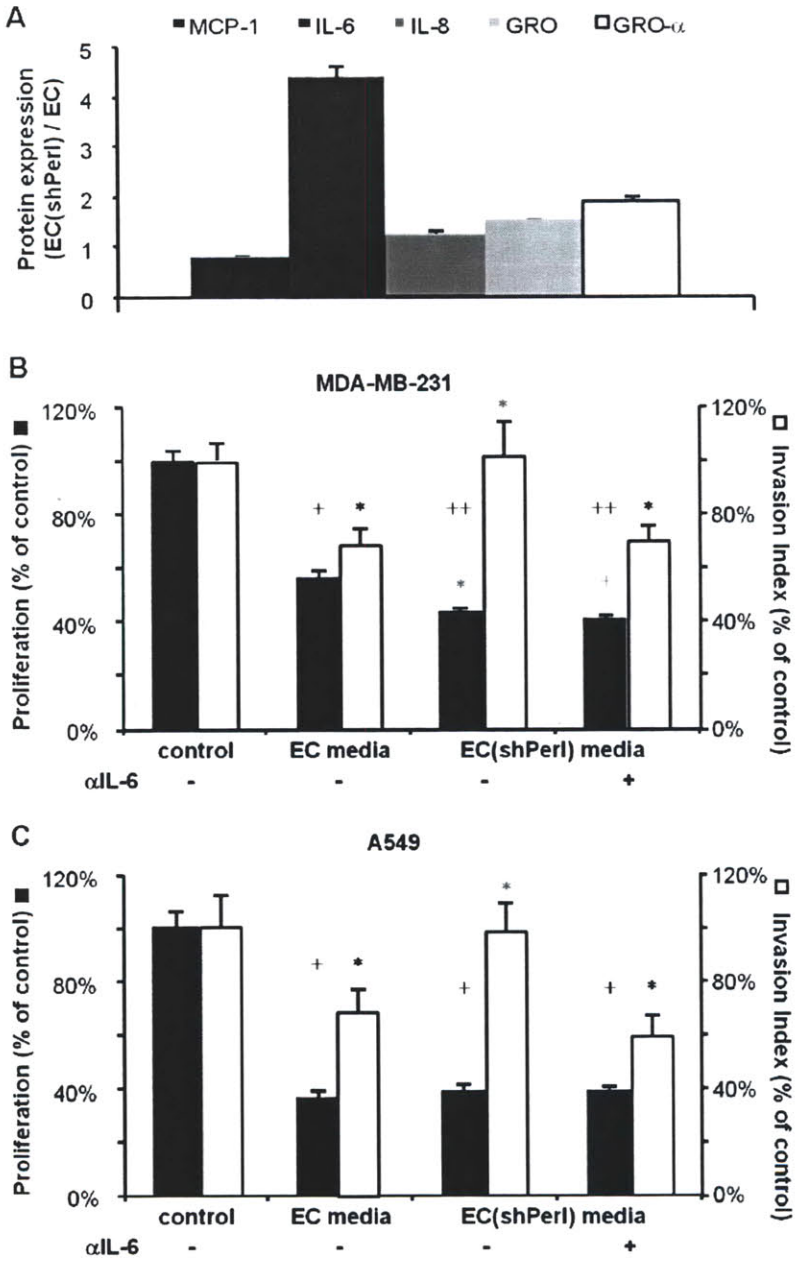

Figure 2.10. Perlecan knockdown abrogates EC suppression of cancer cell invasiveness via increased IL-6 release.

(A) Quantification of cytokine arrays showing ratios of different cytokines in perlecan-silenced ECs (EC_{shPerl}) versus control ECs. (B and C) Effects of IL-6 neutrlaization (neutralizing antibody, 50 μg/mL) in media conditioned by ECs and EC_{shPerl} on the regulation of proliferation (black bars) and inavsiveness (white bars) of MDA-MB-231 (B) and A549 C(). * $p < 0.05$, $^{+}$ $p < 0.005$, $^{++}$ $p < 0.001$ by t-test (black versus control, gray versus EC media). Error bars show SEM.

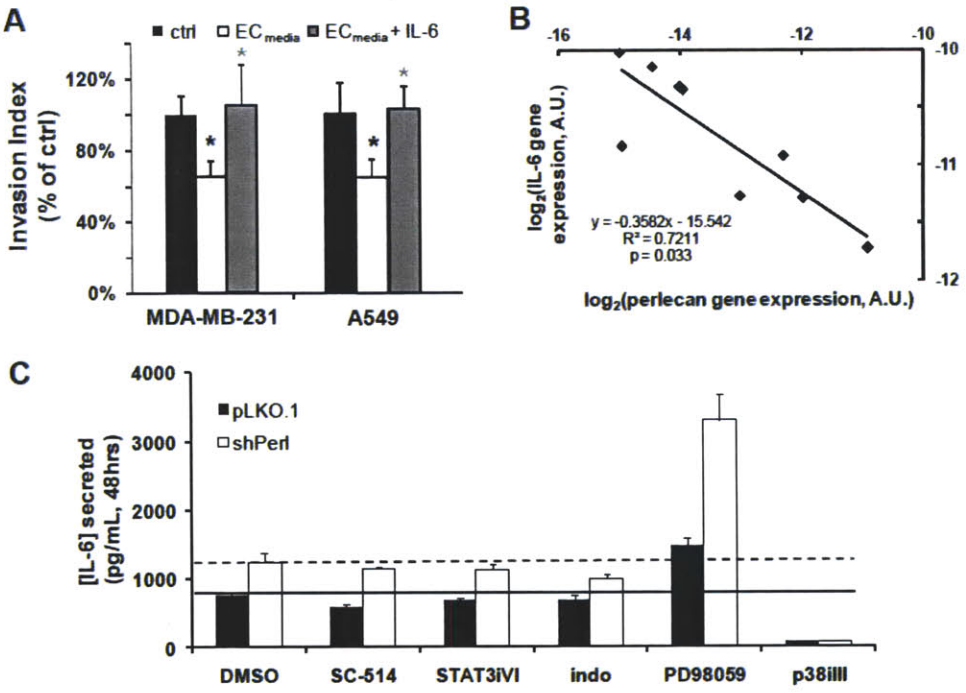

Figure 2.11. Further studies of the perlecan/IL-6 axis in endothelial cells and its role in the regulation of cancer cell invasiveness.

(A) The addition of IL-6 (10 ng/mL) to EC media abrogates its ability to suppress cancer cell invasiveness. (B) IL-6 gene expression increases linearly with perlecan silencing. (C) Inhibiting MEK/ERK signaling (PD98059 (Calbiochem) increased IL-6 secretion in both normal and shPerl ECs, whereas inhibiting p38 MAPK signaling (p38iIII, Calbiochem) decreased IL-6 secretion in both EC(pLKO.1) and EC(shPerl). Inhibition of NF-κB signaling (IKK inhibitor SC-514, Calbiochem), STAT3 signaling (STAT3iVI, Calbiochem), cyclooxygenase activity (indomethacin, indo, Sigma) had minimal effect on IL-6 secretion.

To reduce the likelihood that the perlecan-IL6 EC coregulation arose from off-target effects, we assayed IL-6 gene expression after perlecan silencing using three different perlecan-silencing shRNAs. IL-6 gene expression by qRT-PCR increased directly with perlecan silencing (Fig. 2.11B, $p < 0.05$). We examined further the response to pharmacologic inhibition of pathways relevant to IL-6 expression in ECs transduced with (shPerl) and without (pLKO.1) perlecan-silencing plasmids. Whereas inhibition of p38 MAPK almost completely inhibited IL-6 secretion for perlecan-silenced and control ECs, inhibition of MEK/ERK signaling increased IL-6 secretion more than two-fold (Fig. 2.11C). Inhibition of STAT3, COX, and NF-κB signaling were not as important in this regard.

Matrix embedded ECs suppress xenograft tumor growth.

To understand whether the effects observed on cultured cancer cells could be recapitulated in controlling cancer cells *in vivo* we examined the role of endothelial implants in modulating primary tumor growth. ECs embedded within three-dimensional porous gelatin matrices preserve their phenotype and enable controlled cell implantation in a wide range of models without eliciting an immune response [53, 56-57]. Such matrix-embedded ECs (MEECs) have a similar morphology to ECs cultured on gelatin-coated TCPS (Fig. 2.12A), and provide similar regulation of *in vitro* cancer cell proliferation (Fig. 2.12B) and invasiveness (Fig. 2.12C). Thus, MEECs function as stable, implantable EC constructs useful for studying EC paracrine functions in a wide variety of culture and animal systems.

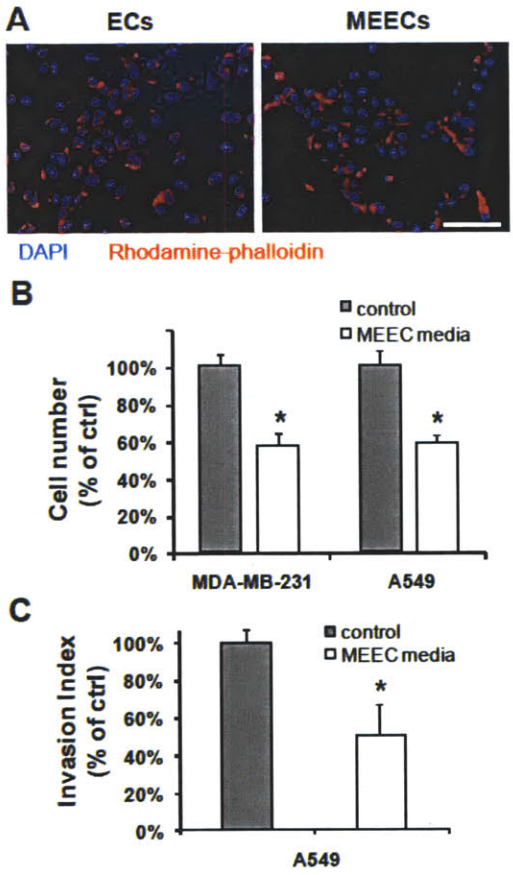

Figure 2.12. MEECs are phenotypically similar to ECs.

(A) ECs and MEECs have a similar morphology, as indicated by their actin cytoskeletal structure (scale bar = 50 μm). MEECs inhibit cancer cell proliferation (B) and invasiveness (C) to a similar extent as do ECs.

MEECs implanted adjacent to established subcutaneous A549 xenograft tumors in nude mice (Fig. 2.13A) reduced tumor growth ($p < 0.05$, Fig. 2.13B). Tumor growth inhibition correlated with a 46±15% decrease in the fraction of Ki-67$^+$ cancer cell nuclei within the tumor ($p < 0.05$, Fig. 2.13C; Fig. 2.14A) and with a 55±21% decrease in the fraction of the tumor filled with cysts ($p < 0.05$, Fig. 2.13D; Fig. 2.15). In addition, p-S6RP levels were reduced by 34±2% in the A549 cancer cells of xenograft tumors as in cell culture ($p < 0.001$, Fig. 2.13E; Fig. 2.14B). By the end of the experiment implanted MEEC constructs had almost completely degraded. There was no evidence that MEECs invaded any of the tumor or that tumor cells occupied any of the microscopic remnants of the implanted matrices.

Cancer cells preconditioned with EC media are less metastatic in vivo.

Because perlecan was important for regulation of cancer cell invasiveness *in vitro*, we examined the role of EC perlecan expression in controlling experimental metastasis. Exponentially growing A549 lung carcinoma cells were injected into the tail veins of nude mice after culture for 4 days in unconditioned media or in media conditioned by either unmodified ECs or perlecan-silenced ECs. Lung mass increased significantly in mice injected with A549 cancer cells cultured in unconditioned media compared with non-tumor-bearing mice 22 days after injection, but not when the A549 cells had been exposed to EC-conditioned media (Fig. 2.16A): This protection was lost when A549 cells were injected after exposure to EC whose perlecan had been silenced. These findings correlated with immunofluorescent staining for the injected A549 cells (Fig. 2.16B): The degree of pulmonary metastasis of A549 cells as defined by a staining

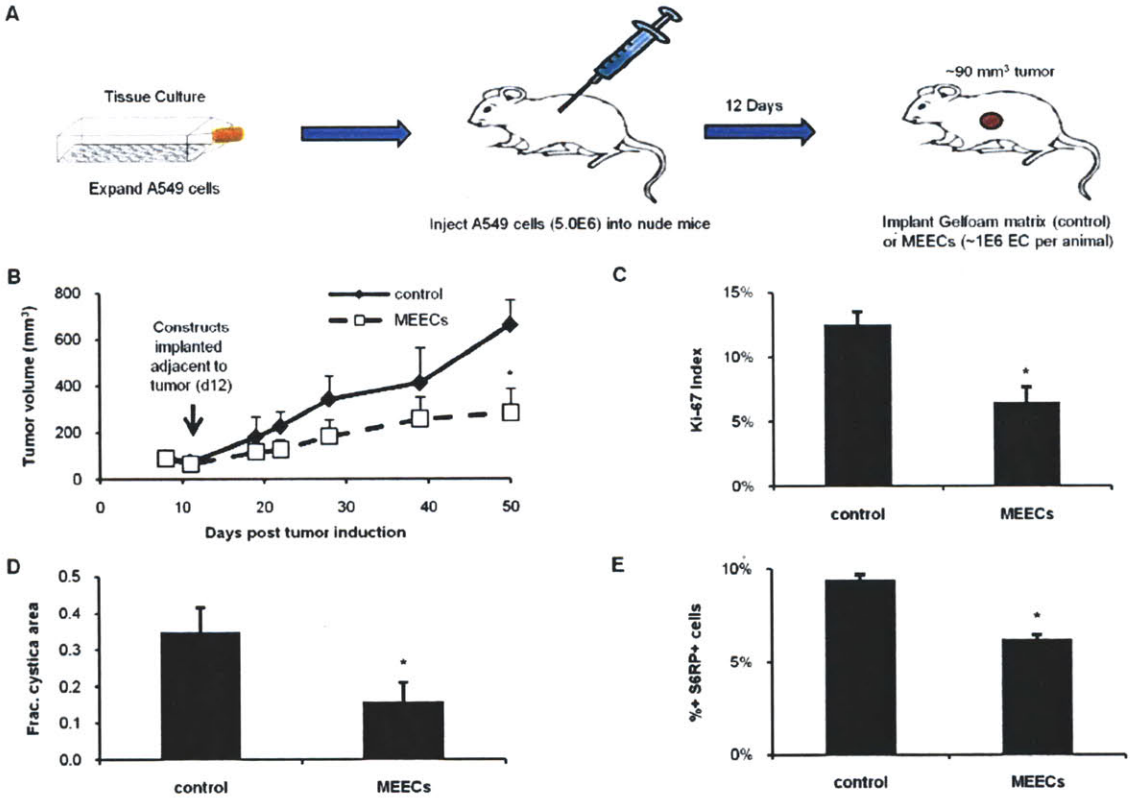

Figure 2.13. Implantation of matrix-embedded ECs (MEECs) adjacent to xenograft tumors reduces tumor growth and aggressiveness.

(A) Schematic of xenograft tumor model with adjacent MEEC implantation. (B) Kinetic growth curves for A549 xenograft tumors in nude mice with control (acellular matrix) or MEEC implants. (C to E) Ki-67 percent nuclear staining (C), cystic mass fraction (D), and p-S6rp percent staining (E) of tumor parenchyma in the above groups. * $p < 0.05$ versu control group by t-test. Error bars show SEM.

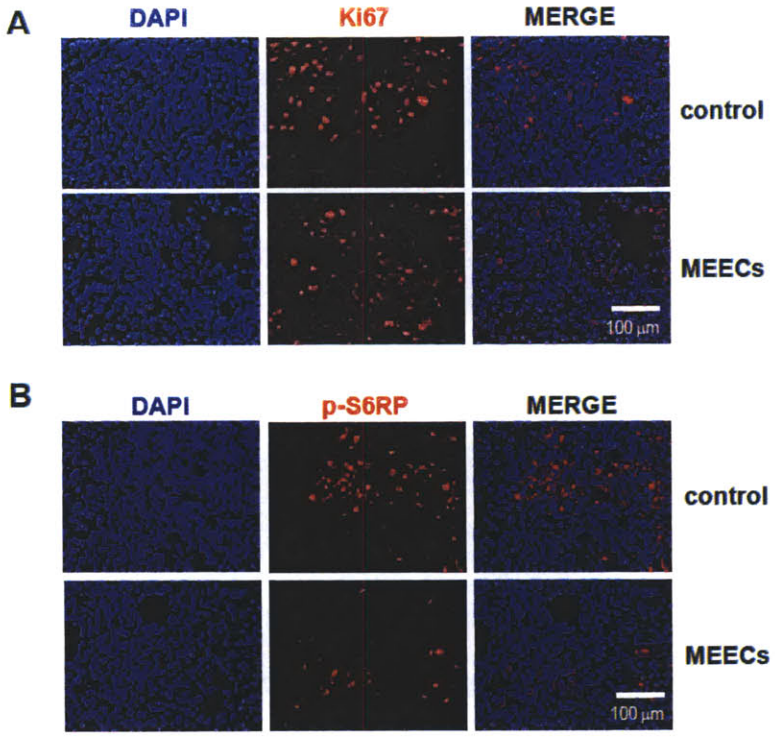

Figure 2.14. Representative Ki67 (A) and p-S6RP (B) staining in control and MEEC-treated A549 xenograft tumors.

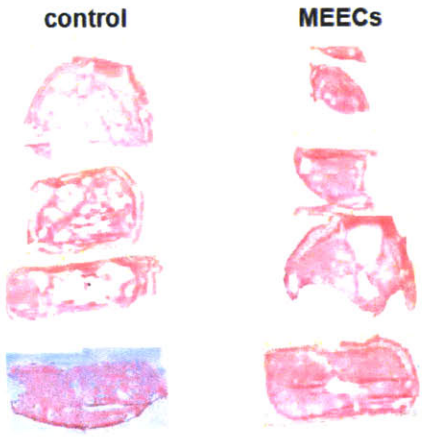

Figure 2.15. H&E-stained sections from control and MEEC-treated tumors showing intratumoral cysts.

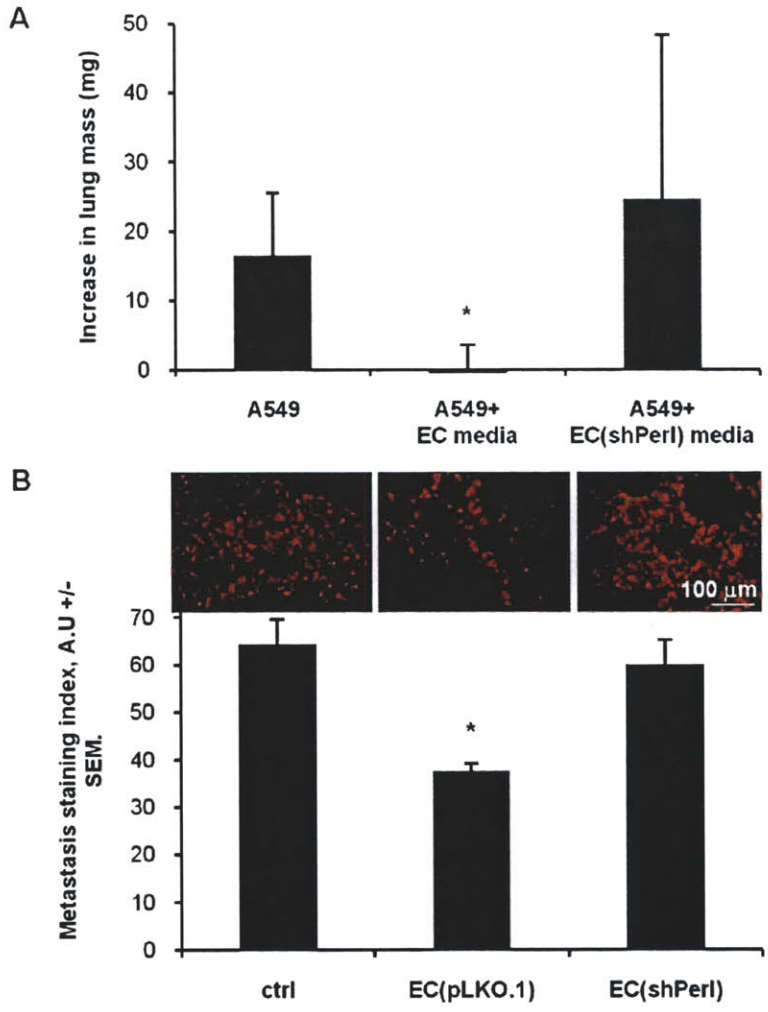

Figure 2.16. A549 cells cultured in media conditioned by intact ECs, but not perlecan-silenced ECs, were less metastatic than control cells.

(A) Increase in lung weights, relative to tumor-free animals, of A549 cells cultured for 4 days in unconditioned (control) media, media conditioned by intact ECs, and media conditioned by perlecan-silenced ECs (EC_{shPerl}). (B) Metastatic index (see Materials and Methods) of lung cryosections in the above groups. * $p < 0.05$ versus control group by t-test. Error bars show SEM.

index was 41±6% lower in mice injected with cancer cells exposed to intact ECs than animals injected with cancer cells cultured in unconditioned media ($p < 0.05$). Similar to the lung mass increase, metastasis inhibition was lost in the EC_{shPerl} media-precultured group. Thus, EC regulatory effects *in vivo* are consistent with our *in vitro* findings.

Taken together, these data suggest that ECs suppress cancer cell proliferation (tumor growth) and invasiveness (metastasis) in a manner highly dependent on the quiescent and intact endothelial phenotype. When EC phenotype is disrupted, there is concomitant alteration of EC cancer-regulatory effects.

Discussion

The vasculature is essential to cancer biology, ensuring perfusion of the tumor mass [63] and control of the biophysical microenvironment [67]. ECs line all vessels and their integrity is critical to vascular health [27, 29, 31, 98-99, 115]. Quiescent ECs suppress every phase of vascular disease, including degree of injury, exposure to toxic products, local thrombosis, inflammation, proliferation, and matrix remodeling. Injured or dysfunctional ECs can promote these events [127]. We now report that quiescent ECs release factors that suppress cancer proliferation and invasiveness *in vitro*. Moreover, perlecan silencing significantly altered EC-mediated regulation of cancer cell phenotype. In addition, the implantation of ECs supported within 3D porous gelatin matrices adjacent to murine xenograft tumors limited primary tumor growth, and preculturing cancer cells with EC secreted factors reduced their metastatic capacity in an experimental metastasis model. Together, our studies support the concept of ECs as paracrine cancer regulators and add depth to the paradigm of tumor angiogenesis by showing how EC-derived signals can directly regulate tumor parenchyma.

The concept of bidirectional EC-tumor interactions can be found in the earliest work on tumor angiogenesis [65]. Increasingly, it is postulated that the complex interplay between tumors and their vasculature depends on more than perfusion alone and that EC-controlled paracrine, or "angiocrine," modes of regulation must be considered [79]. Contact-dependent interactions between the EC-surface receptor DARC and the carcinoma cell-surface receptor KAI1 induce carcinoma cell senescence and thereby reduce metastasis [74]. Subsets of brain vasculature ECs maintain the stem cell

compartment of brain tumors through contact dependent and independent means [75]. We now describe how quiescent ECs regulate cancer cell behavior *in vitro* and control tumor growth and metastatic potential of carcinoma cells *in vivo*. These endothelial effects were not recapitulated by isolated EC-secreted factors or by other stromal cells. Fibroblasts and leukocytes, including myeloid and lymphoid cells, serve initially as tumor-suppressive or permissive regulators, but can be converted into cancer-stimulatory cells [90]. Indeed, ECs, but not fibroblasts, inhibited the proliferation and *in vitro* invasiveness of two distinct cancer cell lines, and the intact EC secretome was more physiologically relevant than a proven clinical cancer chemotherapeutic agent. Rapamycin completely inhibited S6RP phosphorylation, but only modestly curbed cancer cell growth and exhibited none of the anti-inflammatory effects of intact ECs. Moreover, unlike directed pharmacologic effects that target specific pathways, ECs reduced invasiveness of two different cancer cell lines by potentially different means – in MDA-MB-231 through downregulation of metalloproteinases, and in A549 through upregulation of metalloproteinase inhibitors. The limitations of rapamycin's effects validate the coordinated involvement of multiple critical pathways in the regulation of cancer by ECs, and the divergent effects of ECs on matrix remodeling genes speaks to the likelihood of diverse mechanisms controlling different cancer cells. Cell-embedding within matrices enables implantation of intact ECs that possess a broader regulatory potential than isolated pharmacological agents.

The results with EC perlecan silencing illustrate further the complex cellular crosstalk involved in regulating cancer behavior. EC-conditioned media contains several distinct

molecules that likely synergistically regulate cancer phenotype. The heparan sulfate proteoglycan perlecan binds to and mediates the biochemistry of many extracellular matrix components, growth factors, and cytokines [47-48]. Perlecan silencing entirely eliminates EC secretome-mediated inhibition of cancer invasiveness with a more modest effect on growth, remarkably reminiscent of the role of ECs in vascular repair. ECs lacking perlecan expression lose the ability to inhibit thrombosis with a more modestly reduced ability to inhibit hyperplasia [47]. The effects of perlecan modulation speak to the complexity of EC control over cancer and vascular biology and validate the idea that intact cells can restore physiologic balance more readily than a single pharmacologic compound alone. Perlecan knockdown increases EC secretion of several cytokines, including IL-6, but as with perlecan, IL-6 alone cannot explain fully the effects of ECs on cancer cells. Antibody neutralization of IL-6 restored the ability of EC_{shPerl} to inhibit cancer cell invasiveness but had no effect on cancer cell proliferation, and the addition of IL-6 to EC media had the same effect as EC perlecan silencing. Our results corroborate the reported pro-metastatic effect of IL-6 [126] but may indirectly contrast with work showing perlecan depletion (albeit in cancer cells, not ECs) slows tumor growth and reduces metastasis [128-130].

The *in vivo* validation of the EC regulatory effects brings together many of the cell culture and gene expression findings on isolated cancer cells. We generated matrix-embedded ECs (MEECs) by culturing ECs within compressed gelatin matrices. Implanted MEECs slowed the growth, reduced intratumoral cyst formation, and muted the pro-growth signaling within subcutaneous A549 lung carcinoma xenograft tumors in

nude mice when implanted adjacent to tumors. Preconditioning of A549 lung carcinoma cells by culture in EC media substantially reduced their capacity to invade and colonize the lungs of experimental animals, but not if EC perlecan expression was silenced. ECs within the three-dimensional structure of the porous matrices adopt a phenotype that remains stable for months and can be readily implanted within a range of animal models to regulate tissue repair [31, 47, 99, 131]. Allogeneic and even xenogeneic MEEC implants are effective tissue regulators in animals and humans that do not engender an immune response because of the nature of the matrix substratum that supports the embedded ECs [51, 57, 132]. Allogeneic EC constructs from a single host allow formulation of unit doses that have a prolonged shelf-life, are immediately effective upon implantation without need for cell entraining, present consistent biosecretory profiles from sample to sample within a lot [99], and consistent results from patient to patient [60]. Matrix embedded allogeneic EC implants prolonged vascular access graft survival with minimal immune response and fewer adverse effects than acellular control matrix implants [132]. One could well envision that MEEC could be used to reduce tumor size pre-excision, sensitize tumors to chemotherapy and radiation and limit tumor metastasis and recurrence post-excision.

Potential limitations in this study provide insight into avenues for future work. Our "experimental metastasis" model in immunocompromised mice is widely used [118] and enabled examination of the metastatic potential of the same cell lines studied *in vitro,* but lacks the spontaneous detachment from a primary tumor and extravasation into the circulation seen in spontaneous metastasis. We must also consider that as with other

stromal elements that regulate cancer cell proliferation and/or invasiveness, there may be the potential for a conversion to cancer stimulation [89] as the cancer cells evolve to dominate the stroma. Quiescent ECs promote homeostasis, but ECs that are exposed to high concentrations of inflammatory mediators in the tumor milieu [86] may lose this regulatory ability or even promote tumor growth or metastasis. The elevations in IL-6 seen in the tumor microenvironment [122, 126, 133] may act on the ECs and other stromal elements as well as the cancer cells themselves. This idea fits with the recent observation that chemotherapy can stimulate EC IL-6 secretion to create a pro-lymphoma niche [134]. Such observations clearly require that in the future we distinguish between quiescent ECs and those harvested directly from tumors or cultured *in vitro* in a tumor-like environment [100-102].

Our results suggest the coordinate involvement of multiple EC-secreted factors in the regulation of cancer cell biology that requires a whole-cell perspective for a complete appreciation and treatment of neoplastic diseases. The potential of ECs to regulate cancer biology likely transcends their structural roles in tumor vascular conduits and begs further study. In addition, embedding ECs in gelatin matrices enables us to examine the tumor-regulatory impact of ECs from a range of sites and spectrum of differentiation, and/or exposed to a series of pretreatments and altered environments that include the tumor milieu. The role of proteoglycans such as perlecan and cytokines such as IL-6 illustrate complexities in understanding the mechanisms of EC regulation of cancer cell phenotype. Future work will undoubtedly refine our observations, help delineate direct effects on tumor parenchyma from indirect effects on stroma, speak to

the impact of specific signaling pathways, and determine how understanding of EC-cancer crosstalk will aid in cancer diagnosis, prognosis, and treatment. The confluence of emerging elements in cancer biology and tissue engineering holds great promise for the future control of neoplastic diseases.

CHAPTER 3: DYSFUNCTIONAL ENDOTHELIAL CELLS DIRECTLY STIMULATE CANCER INFLAMMATION AND METASTASIS

Abstract

Although endothelial cells have for the most part been viewed as the lining of tumor-perfusing tubes, and their roles as context-dependent regulators of vascular repair have been established, their privileged roles as paracrine regulators of tumor progression has only recently become widely appreciated. In this study we examined how quiescent and dysfunctionally activated endothelial cells differentially regulated cancer cell aggressive properties and inflammatory signaling. We found that while quiescent endothelial cells restrained cancer proliferation and invasiveness and induced controlled inflammatory signaling changes, dysfunctional endothelial cells strongly stimulated cancer inflammatory signaling and invasiveness. We also found that implanted matrix-embedded dysfunctional endothelial cells stimulated spontaneous metastasis while slowing net primary tumor growth. These studies may help to resolve questions as to why anti-angiogenic therapies have mixed effects and may lead to increased understanding and control of the tumor microenvironment.

Introduction

The role of endothelial cells (ECs) in cancer has been primarily considered to be the structurally dysregulated lining of tumor-perfusing blood vessels [63, 67]. The modest [81], or perhaps negative [82-83], efficacy of anti-angiogenic therapies in several types of cancer is a testament to the complexity of EC-cancer paracrine crosstalk interactions. Although there is a rich literature relating to the context-dependent paracrine regulatory roles of ECs in inflammation and vascular disease and repair, and some have in the past considered paracrine EC-cancer regulation [65, 74-76], the concept of "angiocrine" regulation of the tumor microenvironment has been infrequently examined and only recently been formally proposed [79]. Many abnormalities in the tumor EC phenotype relative to that of quiescent ECs have been examined. An "activated" integrin expression pattern [69], dysregulated leukocyte adhesion [70], abnormal responses to oxidative stress[71], and abnormal mechanosensing [72]. These all bear a striking resemblance to the phenotypes of "dysfunctional" ECs identified in a variety of other disease states [17, 73].

We recently showed that healthy, reparative ECs inhibited *in vitro* cancer proliferation and invasiveness, and *in vivo* tumor growth and metastasis [61]. Furthermore, we showed that a controlled disruption of the endothelial phenotype – silencing of the gene encoding perlecan, a heparan sulfate proteoglycan critical for endothelial inhibition of thrombosis after vascular repair [47] – eliminated the ability of ECs to inhibit cancer invasion and metastasis. Brantley-Sieders et al recently showed that endothelial Slit2 expression was critical for tumor suppression and that its inhibition

by EphA2 in tumors was tumor-stimulatory [80]. Thus, just as in vascular disease and repair, ECs are capable of inhibiting tumor aggressiveness, but this inhibitory phenotype can be changed to a tumor-stimulatory phenotype.

In this study we generated *in vitro* dysfunctionally activated ECs (DECs), pathologically deranged by a combination of pro-inflammatory and cancer-derived stimuli. We found that DECs kill cancer cells *in vitro* while simultaneously stimulating cancer inflammatory signaling and invasiveness. These effects correlated with stimulation of spontaneous lung metastasis and local tumor regrowth by matrix-embedded DECs in mice. These studies should help to expand the angiocrine tumor-regulatory paradigm and help to define analogies between EC paracrine regulatory mechanisms in the fields of non-malignant vascular disease and tumor biology.

Materials and Methods

Reagents

Antibodies to NF-κB p65 and p-p65, integrin β3, VE-cadherin, p-TIE2, p-STAT3, cleaved PARP, fibrillarin, GAPDH, β-actin, and α-tubulin were from Cell Signaling Technology. Antibodies to perlecan, VE-cadherin, and NF-κB p65 (immunofluorescence) and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology. The antibody to eNOS was from Abcam. Fluorescently-labeled secondary antibodies and calcein-AM were from Invitrogen. Recombinant human TNF-α was from BioLegend; VEGF and FGF2 were from Invitrogen.

Cell culture

A549, NCI-H520, HOP62, and HOP92 (lung carcinoma) cells (ATCC) and THP-1 (monocytic leukemia) cells (ATCC) were cultured in RPMI with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin on TCPS dishes. from cancer cells was Primary human umbilical vein ECs (HUVECs, Invitrogen) were cultured in EGM-2 (Lonza) with an additional 3% FBS on gelatin-coated TCPS plates and used between passages 3-6. Cells were passaged by detachment with trypsin and split 1 to ~5. EC-conditioned media were generated from confluent HUVEC monolayers by 48 hours of culture in MCDB131 (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells and debris were removed by centrifugation (5 minutes, 500g) and media were aliquotted and stored at -80°C. Cells were cultured under standard conditions (37°C, 5% CO_2). Conditioned medium (48 hr, 6 mL EGM-2 per plate) from cancer cells was pooled and used to replace 33% of the volume of EGM-2. On top of

this, 10 ng/mL TNF-α, 10 ng/mL VEGF, and 1 ng/mL FGF-2 was added to the "dysfunctional" EC growth medium. This is a modification (by addition of the TNF-α) of a published protocol used to compare gene expression profiles of surrogate cultured ECs with those harvested directly from tumors [101].

Matrix-embedded EC (MEECs) were generated by culturing ECs within sterile Gelfoam compressed matrices (Pfizer, New York) [31]. Matrices were cut into 1.25x1x0.3 cm blocks and hydrated in EC growth medium at 37°C for 2-48 hours. ECs were suspended in medium, seeded onto hydrated blocks and allowed to attach for 1.5 hours, then the contralateral side seeded with an equal number of cells. After additional time for cell engraftment two blocks were added to 30 mL polypropylene tubes containing 6 mL of EGM-2. MEECs were cultured for up to 3 weeks, with media changed every 48-72 hours, under standard culture conditions. Samples from each lot were digested with collagenase (type I, Worthington Biochemicals) and cell seeding efficiency determined with a Z1 Coulter particle counter (Beckman Coulter; Fullerton, CA). Cell viability was assessed by trypan blue exclusion.

Gene expression analysis

Total RNA was purified (RNEasy Mini Plus, Qiagen) and cDNA was synthesized (TaqMan reverse transcription reagents, Applied Biosystems) using 1 μg of RNA per sample. Real-time PCR analysis was performed with an Opticon Real-Time PCR Machine (MJ Research) using SYBR Green PCR Master Mix (Applied Biosystems) and appropriate primers. Gene expression was quantified using the ΔΔCt method, with

either 18SRNA or GAPDH as a housekeeping gene. Primer sequences are listed in Table S1. For Figure 2D, qPCR arrays pre-spotted with primers (Lonza) were used for multiplexed medium-throughput gene expression analysis.

Protein expression analysis

Whole cell extracts were harvested with buffer containing 0.5% Triton X-100, 0.1% SDS, protease inhibitor cocktail (Roche), 2 mM sodium orthovanadate, 50 mM sodium fluoride, and 4 mM PMSF. Nuclear and cytoplasmic protein fractions were obtained as described elsewhere [135]. Protein samples were separated on glycine-SDS gels, transferred to nitrocellulose membranes, immunoblotted with the appropriate primary and HRP-conjugated secondary antibodies, treated with a chemiluminescent peroxidase substrate (SuperSignal West Femto, Pierce). Luminescence was measured by a FluorChem luminometer (Alpha Innotech; CA) and analyzed using ImageJ. A cytokine antibody array (RayBiotech; GA) was used following the manufacturer's instructions for assessment of cell biosecretions. Array luminescence was imaged using a FluorChem luminometer (Alpha Innotech; CA) and quantified using ImageJ.

In vitro cell number, proliferation, apoptosis, tube-forming, permeability, monocyte adhesion, and invasion/migration assays

Cell number: Cells were detached by trypsinization and counted with a Z1 Coulter particle counter (Beckman Coulter; Fullerton, CA). Alternatively, the reduction of MTT reagent (Promega) was monitored according to the manufacturer's instructions.

Proliferation: Incorporation of BrdU was monitored via BrdU ELISA (Cell Signaling Technology) according to the manufacturer's instructions.

Tube forming: 15,000 ECs were seeded in each well of 96-well plate coated with 50 μL of Matrigel (BD Biosciences). After 16-20 hours, tube formation was imaged by phase contrast microscopy. ImageJ was used to quantify tube length in the central low-power field, using at least 4 wells per condition.

Monolayer permeability: ECs were seeded onto transwell inserts (0.4 μm pores, BD Biosciences) and grown to post-confluence in 4 days. After this, the medium in the upper chamber was changed to EGM-2 plus 0.1 mg/mL FITC-dextran (70 kD, Sigma), then medium in the lower chamber was changed to fresh EGM-2, and diffusion was allowed to occur for 1 hour at 37°C, after which the inserts were removed and an aliquot was taken from the lower chamber and fluorescence quantified with a Varioskan Flash plate reader (Thermo), with concentration calculated from a standard curve. Results were normalized per endothelial cell in the control condition.

Monocyte adhesion: ECs were seeded onto gelatin-coated 24-well plates and grown to post-confluence over 4 days. After this, $5x10^5$ calcein AM-labeled THP-1 cells in 100 μL of RPMI were seeded onto the monolayers and allowed to adhere for 1 hour at 37°C. After nonadherent cells were removed by gentle washing with PBS, the monolayers were lysed in 0.1% SDS and the lysates was transferred to an optically neutral 96-well plate. Fluorescent signal was measured with a Varioskan Flash plate reader (Thermo), with background from EC monolayers without THP-1 cell dosing subtracted from all measurements. Results were normalized per endothelial cell in the control condition.

Invasion/migration: Chemoinvasion kits (BioCoat, Becton Dickinson) were used according to the manufacturer's instructions. Invaded or migrated cells adherent to the bottom of the inserts were fixed, stained with DAPI (1 μg/mL, 30 minutes) and imaged by epifluorescence microscopy. Images were analyzed by visual inspection and cytometric quantification of 4 random 20X fields at the microscope or by using the "particle counter" feature of ImageJ for the central 10X field. Data are expressed as an invasion index [117], the average number of invaded cells and the average number of migrated cells of a given condition, normalized to the control condition with at least 3 wells used per condition.

Murine tumor model

All *in vivo* experiments were approved by the Massachusetts Institute of Technology Committee on Animal Care and comply with NIH guidelines.

Female C57BL6 mice, aged ~6 weeks, were purchased from Jackson Laboratories and used in the Lewis lung carcinoma (LLC) implantation-resection-metastasis model [136]. Lewis lung carcinoma cells (ATCC) were passaged serially through mice for at least 2 generations before use in this model to ensure high metastatic capacity. $1*10^6$ LLC cells were injected (suspended in 100 μL HBSS after harvesting and rinsing 2X with HBSS to remove serum and trypsin) subcutaneously on the dorsal surface. After allowing 6 days for tumor growth, either acellular Gelfoam (control), MEECs (~1E6 cells per animal), or dysfunctional MEECS (D-MEECs, ~1E6 cells per animal) were washed twice with HBSS and implanted adjacent to the tumors, which were all approximately

1000 mm^3 in volume. The surgical site was sealed with tissue clips. After 8 additional days, the tumors and implants were excised and the surgical site was again sealed with tissue clips. 14 days after the resection, the animals were sacrificed by CO_2 inhalation. All surgeries were performed under anesthesia with 2% isoflurane delivered via nose cone and 0.1 mg/kg buprenorphine administered perioperatively. Tumors were measured 1-2 times/week with Vernier calipers, using two orthogonal measurements to estimate the volume, assuming a prolate spheroid geometry.

Results

In vitro dysfunctionally activated endothelial cells (DECs) have dysregulated phenotypes.

We generated a endothelial cell phenotype that exhibits increased permeability, dysregulated proliferation and angiogenesis, and leukocyte adhesion – similar to the classic descriptions of dysfunctional ECs (DECs) [17, 137] [138]. DECs grew more slowly in culture compared to ECs, with 42±2% fewer cells at confluence than control ECs ($p = 0.002$, Fig. 3.1A) and accordingly correspondingly larger cells. Immunofluorescent staining of the ECs and DECs revealed morphologic abnormalities in DECs. DECs were larger and exhibited a spindly fibroblast-like morphology, compared to the typical cobblestone morphology of postconfluent ECs (Fig. 3.2B).

Their ability to form tubes on Matrigel was reduced by 26±9% ($p = 0.007$, Fig. 3.1B). More striking was the dramatically increased cell-normalized permeability (2.5±0.4 fold $p = 0.036$, Fig. 1C), and 4.4±0.3 fold increased avidity for binding THP-1 monocytes ($p = 3.5*10^{-7}$, Fig 1D). Indices of endothelial activation were increased and indices of quiescence were reduced (Fig 3.2A). DECs possessed, relative to control ECs, increased phosphorylation of VEGFR2 (8.7±2.6 fold, $p = 0.022$) and total amount of integrin β3 (2.3±0.4 fold, $p = 0.023$) and decreased VE-cadherin (39±12%, $p = 0.04$), eNOS (92±1%, $p = 8.5*10^{-6}$), and phosphorylated Tie2 (81±7%, $p = 0.009$).

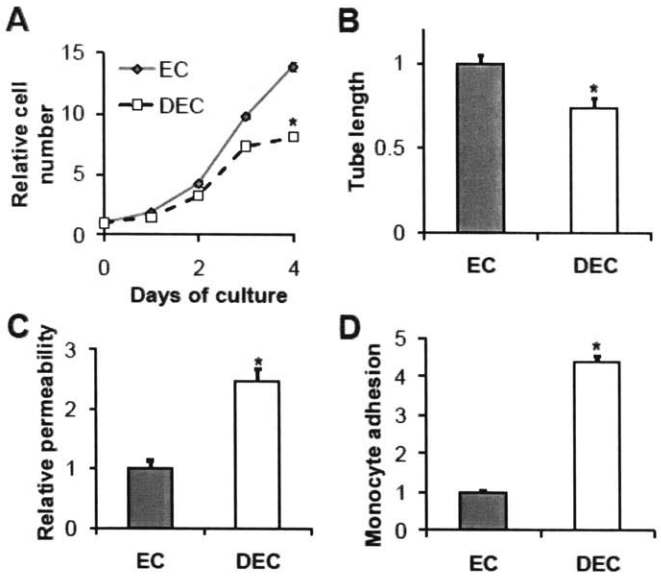

Figure 3.1. The in vitro "dysfunctional" EC (DEC) phenotype includes dysregulated proliferation, tube formation, high permeability, and avid monocyte binding.

(A) Growth curve of endothelial cells cultured under normal ("EC") or dysfunctional ("DEC") in vitro conditions. (B) Tube length per field of ECs and DECs after 2 days of differential culture. (C) Permeability to the passage of FITC-dextran of confluent EC or DEC monolayers after 4 days of differential culture. (D) Adhesion of THP-1 monocytic leukemia cells to confluent EC or DEC monolayers after 4 days of differential culture. * $p < 0.05$ versus EC by t test.

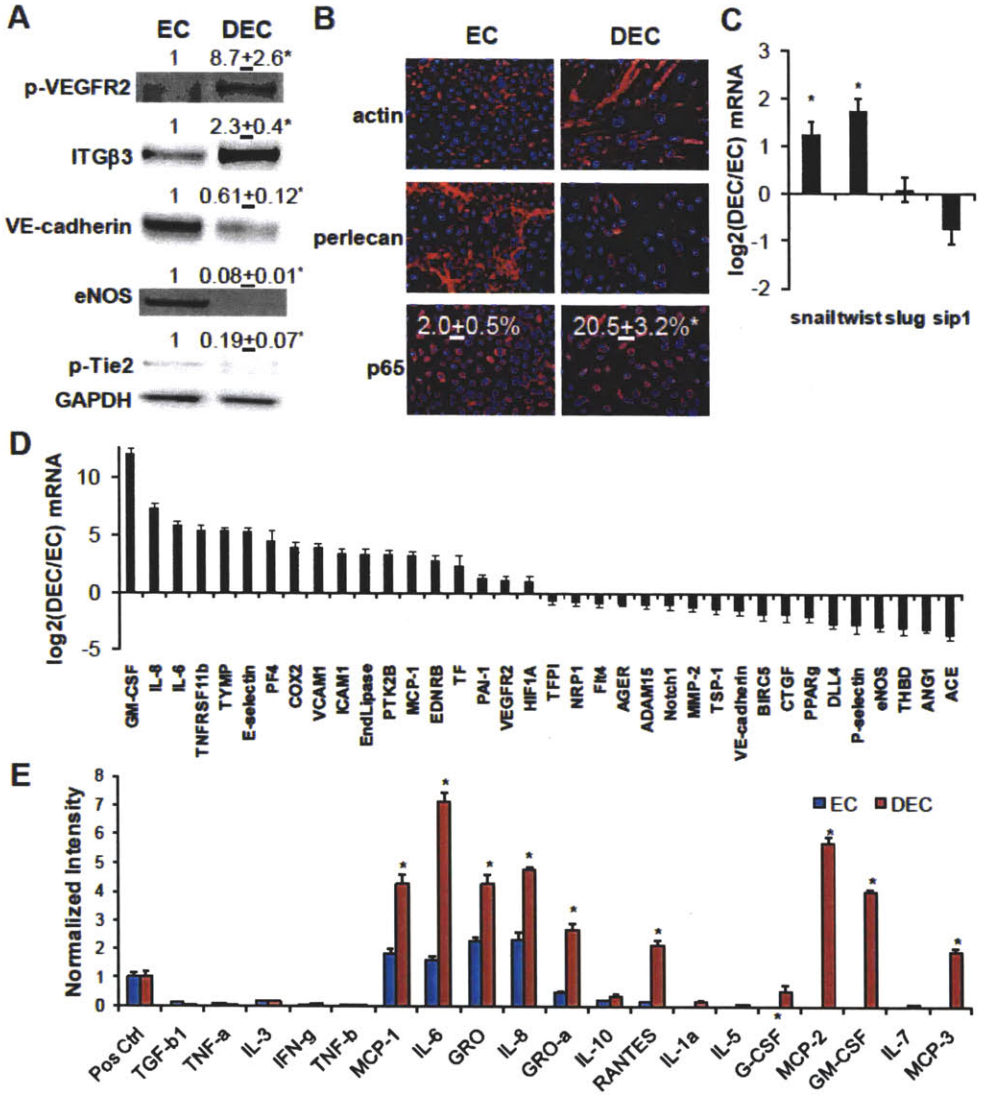

Figure 3.2. The DEC phenotype manifests in increased dysfunctional indices, reduced quiescent indices, altered and inflamed morphology, and highly pro-inflammatory and pro-thrombotic expression profiles.

(A) Western blot of whole cell lysates of ECs and DECs, with quantification shown for the DEC relative to the EC. (B) Immunofluorescent staining for actin (fluorescent phalloidin), perlecan, and NF-κB p65 in ECs and DECs. Nuclei are labeled in blue (DAPI). (C) qRT-PCR analysis of endothelial-mesenchymal transition transcriptional activators. (D) qRT-PCR array analysis of endothelial inflammatory, thrombotic, and quiescent differentiation genes. (E) Cytokine dot blot of EC and DEC secretions. * $p < 0.05$ by t test.

DECs showed a roughly 10-fold increase in the percentage of cells with NF-κB p65 nuclear localization after 4 days of culture ($p = 0.005$, Fig. 3.2B), indicating a sustained, persistent dysfunctional state. These results were verified by examination of the nuclear and cytoplasmic protein fractions from ECs and DECs (Fig. 3.3): DEC nuclei contained more NF-κB p65 and STAT3β than EC nuclei. Additionally, the DECs showed very low expression of intact perlecan (Fig. 3.2B), which we have previously shown to be important for maintaining the quiescent EC phenotype [47, 61].

Gene expression profile of DECs.

Since the endothelial-to-mesenchymal transition (EndMT) can cause ECs to adopt a fibroblast-like morphology and may be of direct relevance in several inflammatory and tumor-associated states [85, 139-141] we examined gene expression changes of EndMT-associated transcription factors in DECs. We found (Fig. 3.2C) approximate 3-fold increases in the levels of Snail and Twist transcripts ($p < 0.05$ for each), and using qRT-PCR arrays, we identified widespread changes in the DEC transcriptome, relative to EC controls (Fig. 3.2D). The expression of many pro-inflammatory NF-κB target genes – e.g. GM-CSF, IL-8, IL-6, and E-selectin – were significantly increased, the expression of quiescence-promoting, anti-inflammatory genes – e.g. eNOS, VE-cadherin, Ang1 – were decreased, the expression of leukocyte adhesion molecules VCAM1 and ICAM1 were increased, and the balance of coagulation-related genes was shifted toward a pro-coagulant state (higher tissue factor, TF, and lower thrombomodulin, THBD). Analysis of the protein secretions by cytokine blots (Fig. 3.2E)

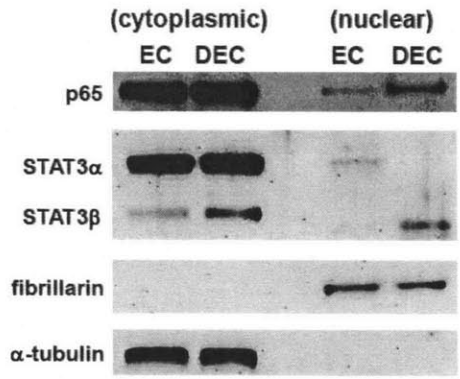

Figure 3.3. DEC nuclei contain more NF-κB p65 and STAT3 than EC nuclei.

Cytoplasmic and nuclear protein fractions of ECs and DECs, with α-tubulin serving as a cytoplasmic loading control and fibrillarin as a nuclear loading control.

of ECs and DECs corroborated and extended the gene expression changes that we identified: DECs showed large increases in secretion of pro-inflammatory cytokines MCP-1, IL-6, IL-8, GRO-α/CXCL1, RANTES, G-CSF, MCP-2, GM-CSF, and MCP-3. Importantly, although the culture cocktail used to generate "DECs" contained 10 ng/mL TNF-α, this protein was virtually undetectable in DEC-conditioned media, indicating that the exogenous TNF-α was sufficiently removed by washing before conditioned media collection. The other exogenous pro-angiogenic and cancer-secreted factors were also likely similarly removed.

We analyzed the expression of selected genes in ECs treated for four days with individual components of this cocktail to partially decompose their effects on EC gene expression (Fig. 3.4). Replacement of 33% of the culture medium with pooled conditioned media from cancer cells had similar effects to TNF-α (10 ng/mL) alone, and when combined with TNF-α recapitulated most of the effect of the complete cocktail. However, as evidenced for example by eNOS gene expression, the complete cocktail was more effective than its individual components, and more than the combinations of its components. EC phenotype was not dramatically changed by sustained culture in saturating concentrations of VEGF (10 ng/mL) plus FGF2 (1 ng/mL), implying a resistance by postconfluent ECs to pro-angiogenic activation.

Cancer cell proliferation, apoptosis, inflammation, and invasiveness.

We have previously shown that quiescent ECs can release factors that inhibit the proliferation and invasiveness of cancer cells in a manner that correlates with steady-

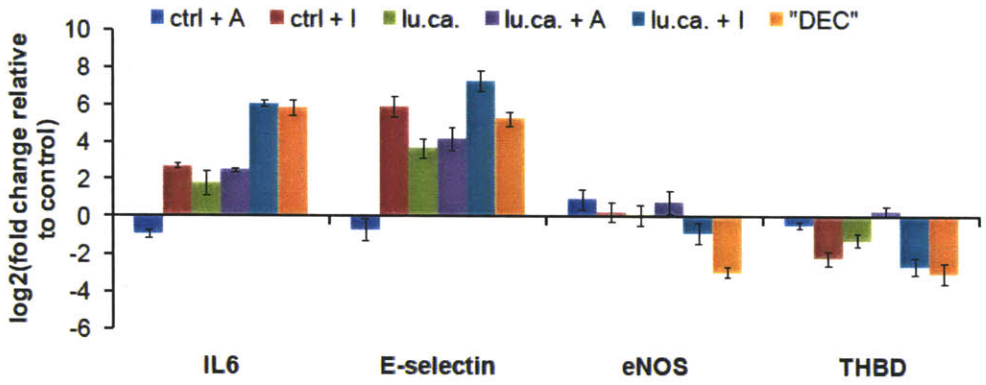

Figure 3.4. The combination of pro-inflammatory, pro-angiogenic, and cancer-derived factors in the "DEC" culture medium act to generate the most deranged EC phenotype.

Gene expression (qRT-PCR) analysis of IL-6, E-selectin, eNOS, and thrombomodulin (THBD) of endothelial cells cultured for 4 days with combinations of 10 ng/mL VEGF + 1 ng/mL FGF2 ("A"), TNF-α (10 ng/mL) ("I"), and lung cancer conditioned media (33% v/v) relative to endothelial cells cultured under standard conditions. "DEC" denotes the use of the full cocktail, containing VEGF, FGF2, and TNF-α.

state inhibition of pro-inflammatory signaling and that targeted disruption of the quiescent phenotype could impact EC regulation of cancer [61]. Since the DEC phenotype was highly deranged and pro-inflammatory, we hypothesized that DECs would be cancer-stimulatory along multiple axes.

The growth of three different cancer lines was significantly inhibited by EC media, (40-70% reduction, Fig. 3.5A, $p < 0.003$), and reduced even further by DEC media (~80% reduction, $p < 3*10^{-7}$ for all versus control; $p < 0.04$ for all versus EC media). The inhibition of cancer cell number correlated with a ~20% reduction in proliferation each day for both ECs and DECs (Fig. 3.5B). DECs, unlike ECs, robustly induced apoptosis in A549 cells as measured by Western blot of cleaved PARP, a DNA repair enzyme induced by caspase-3 ($p = 0.013$ versus control, $p = 0.033$ versus EC, Fig. 3.5C). These results correlate with the known pro-apoptotic effects of pro-inflammatory cytokines such as IL-6 [125-126], whose secretion is increased in DECs by ~5-fold relative to ECs (Fig. 3.2E).

The activation of pro-inflammatory signaling in cancer cells has been implicated in many facets of the disease [87, 122, 135], and though pro-inflammatory cytokines such as IL-6 can kill cancer cells, those cells that survive tend to be particularly aggressive and metastatic [126]. Therefore we examined signaling through the canonical pro-inflammatory STAT3 and NF-κB pathways in A549 lung carcinoma cells after variable time in culture in EC or DEC-conditioned media (Fig. 3.6A). DECs caused a 6.2 ± 0.8 fold increase ($p = 0.0006$) in STAT3 phosphorylation at 24 hours, which inverted to a $79\pm3\%$

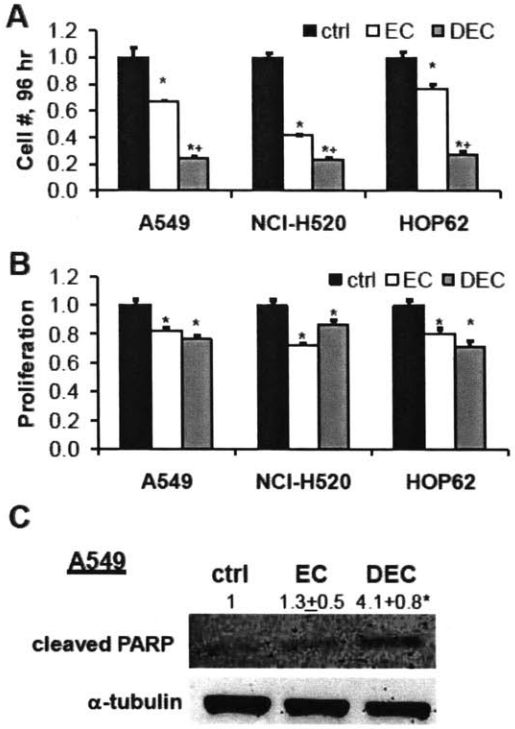

Figure 3.5. ECs inhibit cancer cell proliferation, but pro-inflammatory DECs kill cancer cells.

(A) MTT assay of the A549, NCI-H520, and HOP62 lung carcinoma cells after 4 days of culture in either unconditioned (control), EC-conditioned, or DEC-conditioned media. (B) BrdU incorporation of the same cells during hours 24-48 of culture in the same sets of conditioned media. (C) Western blot of cleaved PARP in A549 cells 4 hours after initiation of culture in the same sets of conditioned media. * $p < 0.05$ versus control, $^{+}$ $p < 0.05$ versus EC by t test.

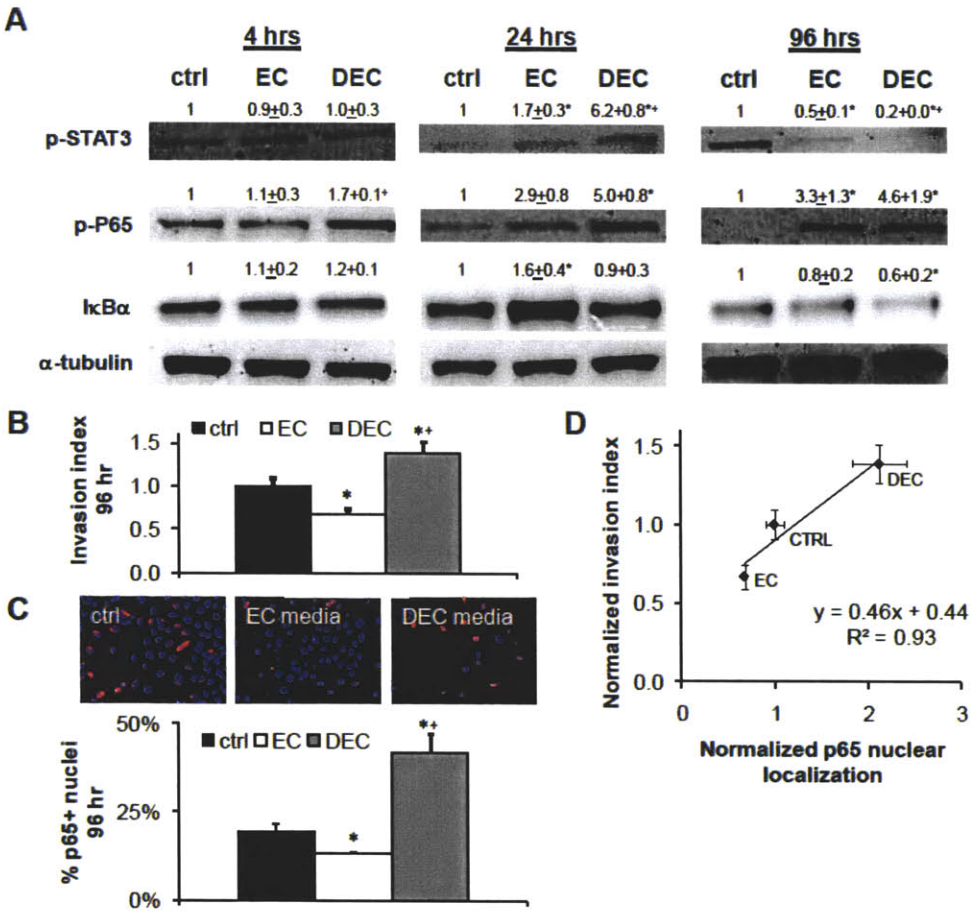

Figure 3.6. ECs control lung cancer inflammatory signaling and reduce invasiveness, whereas DECs robustly stimulate lung cancer inflammatory signaling and stimulate invasiveness.

(A) Time course of STAT3 and NF-κB pathway activity induced by either unconditioned (control), EC-conditioned, or DEC-conditioned media as assayed by Western blot of whole cell lysates of A549 cells. Quantification, relative to control, is shown above each representative band. (B) In vitro chemoinvasion index of A549 cells after 4 days of culture in the same sets of conditioned media. (C) Immunofluorescent nuclear localization of NF-κB p65 signal in A549 cells after 4 days of culture in the same sets of conditioned media. (D) Correlation between effects of EC or DEC secretions on A549 invasiveness and NF-κB p65 nuclear localization. * $p < 0.05$ versus control, $^{+}$ $p < 0.05$ versus EC by t test.

decrease ($p = 0.0002$) by 96 hours. Secretions from quiescent ECs in contrast caused a small but measurable STAT3 activation in A549 cells at 24 hours (1.7 ± 0.3 fold, $p = 0.049$) that also inverted to a $46\pm14\%$ ($p = 0.037$) decrease by 96 hours. Similarly, there was a 1.7 ± 0.1 fold increase ($p = 0.035$) in NF-κB p-P65 induced by DEC-conditioned media at 4 hours that increased to 5.0 ± 0.8 fold by 24 hours ($p = 0.0046$) and remained elevated at by 96 hours ($p = 0.01$). Here too EC media induced a smaller, more controlled pro-inflammatory response, with a trend toward ($p = 0.075$) increased p-P65 at 24-hrs and moderate sustained activation at 96 hours ($p = 0.0055$). Intriguingly, the observed changes in NF-κB p65 activation caused by EC media were associated with a 1.6 ± 0.4 fold increase ($p = 0.047$) in IκBα at 24 hours, supporting the notion of a controlled or balanced inflammatory activation by EC secretions.

We next hypothesized that the intense stimulation of pro-inflammatory signaling in A549 cells by DEC media would stimulate invasive properties, an *in vitro* correlate of metastasis. We found that media from quiescent ECs inhibited the in vitro invasiveness of A549 cells by $33\pm10\%$ ($p < 0.005$, Fig. 3.6B) and DECs stimulated A549 invasiveness by $39\pm18\%$ ($p < 0.01$, Fig. 4B). These effects correlated directly ($r^2 = 0.93$, Fig. 3.6D) with a $32\pm7\%$ decrease in A549 nuclear NF-κB immunofluorescent staining by culture in EC media ($p = 0.037$, Fig. 3.6C) and a 2.1 ± 0.4 increase by DEC media ($p = 0.022$, Fig. 3.6C).

Taken together, our results suggest a controlled, long-term cancer-inhibitory effect on cancer cells by quiescent ECs and a pro-inflammatory, invasion-stimulatory effect on cancer cells by dysfunctional ECs.

Adjacent D-MEECs stimulate spontaneous metastasis.

To study EC cancer-regulatory phenomena in animals, we chose the Lewis lung carcinoma implantation-resection-metastasis model. This model has the advantage of spontaneous metastasis from an easily accessible primary tumor. We have in the past used surgically implanted matrix-embedded ECs in a variety of disease models, including primary xenograft tumor mouse models [61]. For this experiment we generated matrix-embedded quiescent ECs (MEECs) or dysfunctional ECs (D-MEECs) by prolonged culture in the same cocktail used to we generated DECs for *in vitro* work. D-MEECs phenotype was changed relative to MEEC phenotype in a manner that was qualitatively similar to DECs versus ECs (Fig. 3.7).

D-MEEC implants had significant effects on tumor biology. Primary tumors were smaller, reflecting either retarded proliferation or enhanced killing of cancer cells, and distant metastasis were stimulated. Tumor volumes were equal at matrix implantation but the volumes of tumors with adjacent D-MEEC implants were reduced by 54$\pm$14% relative to the control tumors 14 days later ($p = 0.048$, Fig. 3.8A). The reduction in tumor size in the D-MEEC group correlated with a 68$\pm$10% reduction in Ki67 index ($p = 0.02$, Fig. 3.8B) and a 67$\pm$13% increase in the number of cleaved caspase 3 events per 10X

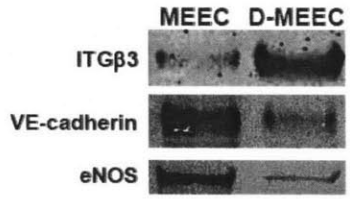

Figure 3.7. Quiescent matrix-embedded endothelial cell (MEEC) phenotype is similar to that of ECs and dysfunctional matrix-embedded phenotype (D-MEEC) is similar to the phenotype of DECs.

(A) Western blot of both cell types for some of the same markers used to characterize ECs and DECs. * $p < 0.05$ versus control.

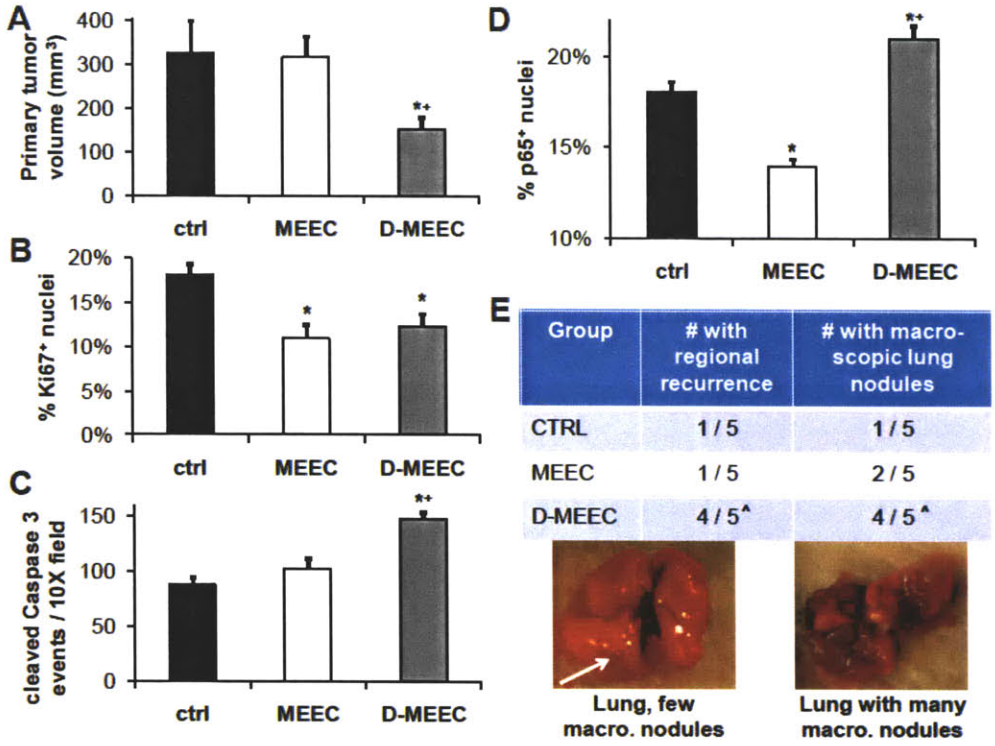

Figure 3.8. Adjacent D-MEECs cause increased spontaneous metastases and thereby slow the net growth of primary tumors.

(A) Volumes by caliper measurements of explanted Lewis lung carcinoma tumors. * $p < 0.05$ versus control by t test. (B) Percent of Ki67-positive nuclei of thresholded immunofluorescent primary tumor cryosection images. (C) Number of cleaved caspase 3 events per 10X field of thresholded immunofluorescent primary tumor cryosection images. (D) Percent of NF-κB p65-positive nuclei of thresholded immunofluorescent primary tumor cryosection images. (E) Table showing fractions of mice with regional (posterior cervical) metastasis and with macroscopic lung metastases 2 weeks after primary tumor resection. * $p < 0.05$ by t test. $^{+}$ $p < 0.05$ by proportion z-test.

field ($p = 0.0001$, Fig. 3.8C). Although we observed no gross effects on Lewis lung tumor growth or metastasis by adjacent quiescent MEEC implants, the MEEC-treated tumors had Ki67 index that was $61\pm9\%$ lower than the control group ($p = 0.006$, Fig. 3.8B).

In addition to markers related to tumor growth, inflammatory markers were also affected by the presence of adjacent endothelial implants. Concomitant with the reduction in primary tumor size caused by D-MEECs, there was a $16\pm5\%$ increase ($p = 0.011$, Fig. 3.8D) in the fraction of nuclei within tumor cryosections that stained positively for NF-κB p65. This increase in inflammatory signaling correlated with an increase in the metastatic properties of the D-MEEC treated Lewis lung primary tumor. Four of five animals had macroscopic lung metastatic lung nodules, and the same animals each had regional cervical metastatic tumors ($p < 0.05$ each by proportion z-test). Only one of five animals in the acellular control matrix group had regional recurrence and one with macroscopically identifiable lung nodules (different animals). Although there was a $23\pm4\%$ reduction ($p = 0.0002$, Fig. 3.8D) in the inflammatory signaling of the MEEC-treated primary tumors, there was no reduction in metastasis relative to the control group in these animals.

In sum these results reinforce the conclusions drawn from the *in vitro* work and demonstrate that biochemically modified "dysfunctional" endothelial cell implants can cause tumors to grow more slowly but metastasize more effectively.

Discussion

The emerging angiocrine paradigm posits that recruited endothelial cells (ECs) stimulate tumor progression [79]. We, on the other hand, have recently demonstrated that quiescent ECs are cancer-inhibitory and that if the quiescent endothelial state is disrupted then the regulatory effects on cancer can change profoundly [61]. Therefore we hypothesized that, similar to state-dependent regulatory effects in vascular disease whereby quiescent ECs suppress diverse disease processes and dysfunctional endothelial cells stimulate the same processes, there is a state-dependent aspect to endothelial cancer regulation. In this study we showed that dysfunctional, pro-inflammatory endothelial cells robustly activate pro-inflammatory signaling within cancer cells in a manner that is correlated with enhanced *in vitro* invasiveness. When dysfunctional ECs were implanted as matrix-embedded engineered cell constructs adjacent to tumors, the rates of spontaneous metastasis of cancer cells from the tumors significantly increased.

We rigorously characterized the isolated phenotype of *in vitro* "dysfunctional" ECs, which largely mimicked the known phenotype of dysfunctional endothelium in atherosclerotic vascular disease [17]. Interestingly, the phenotype of tumor-associated ECs has many features in common with dysfunctional ECs, including activated integrin (e.g. integrin β3) and extracellular matrix expression [69], dysregulated leukocyte adhesion (e.g. E-selectin, VCAM1, ICAM1, etc.) [70], abnormal responses to oxidative stress [71], and abnormal sensation and response to the mechanical microenvironment [72]. These similarities are likely caused by similarly pathologic activating stimuli,

including inflammation, present in both the cancer [88, 96, 122] and the atherosclerotic milieus [103-104]. Thus, we posit that the use of dysfunctional ECs in the cancer domain may be directly relevant.

Our work in examining paracrine effects of dysfunctional ECs adds to a large body of work examining the critical and far-reaching effects of inflammatory signaling as it contributes to tumor progression [88, 96]. The NF-κB [135] and STAT3 [87, 122] signaling pathways are particularly important in controlling many cancer cell-stimulatory pro-inflammatory signals. These pathways are critical for both the cancer cells themselves [135] and the stroma [142] within tumors. Indeed, we saw activation of these pathways in a type of stromal cell (the EC) inducing activation in the parenchymal (cancer) cell. In addition, paracrine signaling dynamics may be important in cancer-stroma crosstalk interactions. For example, NF-κB signaling-induced transcriptional changes are highly context-dependent [143-144] and depend on the relative quantities of interacting proteins such as IκBα [145]. Here, we saw that while quiescent ECs induced a moderate, controlled state of inflammation within target cancer cells resulting in a net decrease in the proliferation and invasion of cancer cells, dysfunctional ECs more efficaciously induced pro-inflammatory signaling in a manner that killed cancer cells, but left behind a particularly aggressive, invasive subset of cancer cells. Therefore it may be that quiescent ECs are not strictly anti-inflammatory, but rather may sometimes induce controlled inflammatory signaling, with balanced action of activating (e.g. p65) and inhibiting (IκBα) components, to control wound healing and cancer inhibition. We have identified distinct and complementary wound healing paracrine roles

for epithelial and endothelial cells in guiding tracheal repair [31]. In addition to the examination of cancer-inhibitory roles of ECs by our laboratory [61] and others [76], investigators have discovered cancer-cytotoxic secretions emanating from differentiated normal epithelial cells [146]. These phenomena illustrate an underappreciated role of the healthy microenvironment in restricting cancer progression [92].

Our dysfunctional ECs secreted large quantities of several pro-inflammatory molecules and many of these could individually cause dramatic direct (targeting the cancer cells themselves) and indirect (targeting the regulatory stroma) cancer cell effects. For example, IL-6 has known metastasis-stimulatory roles [125-126] in addition to its classic immunomodulatory and endothelial activating functions. We have previously shown that IL-6 secretion by endothelial cells can increase when the quiescent phenotype is disrupted, and others have shown that specific stresses, such as chemotherapy [134], can induce IL-6 secretion in a tumor-supporting manner, illustrating the particular importance of endothelial-derived IL-6 within the tumor milieu. The benefit of using intact cells as delivery vehicles for cytokines – as opposed to delivery of isolated factors – is that the cells release these factors in a dynamic manner, in proportions that are physiologically and temporally relevant. A difficulty arises, then, in attributing specific effects to specific secreted mediators. For this reason we did not attempt here to parse the effects of individual secreted molecules like IL-6, since several other molecules like MCP-1 and IL-8 were also upregulated, but rather characterized target cancer cell responses to the combined sets of molecules.

Although the Lewis lung carcinoma model is a heterotopic xenograft model and therefore the primary tumor may experience a somewhat different microenvironment than the lung from which it originated, it has the advantage of easier surgical access and robust spontaneous metastasis [136]. We have used matrix-embedded endothelial cell (MEEC) constructs to enable novel means of studying endothelial paracrine regulatory processes in a wide range of injury [31, 47, 60, 99] and tumor models [61]. In our prior work, we had focused on implanting quiescent MEECs to help guide repair and restrain cancer progression. Here we were able to make the matrix-embedded cell phenotype dysfunctional and found dramatically different effects on primary tumor growth and spontaneous metastasis than those of quiescent endothelial implants. However, in the present study we did not observe inhibition of cancer metastasis by quiescent EC implants, despite a quantifiable molecular/signaling impact (reduced Ki67 and NF-κB p65 nuclear staining) in the primary tumor. In our prior studies [61], multiple weeks were required for quiescent EC implants to begin to affect gross tumor growth rate. Thus, longer exposure to quiescent MEEC implants may allow potential gross anti-cancer effects to emerge. Further, although the magnitude of the stimulation of metastasis caused by adjacent dysfunctional MEEC implants was disproportionate to the more modest increase in NF-κB p65 nuclear staining, it may be that either the most highly stimulated cells had already metastasized or that concomitant signaling changes along other pro-inflammatory axes (e.g. STAT3) may dominate here.

Overall our studies illustrate how the endothelial phenotype can be manipulated to be metastasis-stimulatory and may implicate endothelial dysfunction as a major

contributor to the evolving tumor microenvironment. These results may explain how certain anti-angiogenic therapies that damage the vasculature may help to shrink tumors – in ways independent from perfusion inhibition – but ultimately lead to local invasion and distant metastasis [82-83]. Future work will help to define specifically how various components of the tumor microenvironment can make endogenous tumor endothelium dysfunctional and perhaps be illustrate more similarities between the tumor endothelial phenotype and the atherosclerotic endothelial phenotype. These studies may motivate novel pharmacologic or cell-based interventions designed to modulate such effects to help slow or reverse tumor progression.

CHAPTER 4. CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

List of proposed future experiments

Hypothesis	***Proposed Experiments***	***Expected results***
Secretions from reparative ECs inhibit signaling through critical cancer cell signaling pathways	Using particular oncogene-addicted cancer cells (e.g. EGFR-addicted NSCLC, Braf-addicted melanoma, β-catenin-addicted CRC, Her2-addicted breast carcinoma); use reverse network engineering to analyze transcriptome-wide cancer cell responses	The reparative EC secretome inhibits a broad array of target molecules within cancer cells to "optimally" interfere with cancer cell aggressive properties.
Targeted modulations in EC phenotype will affect cancer-regulatory effects	Modulate *in vitro* EC phenotype by disrupting or overexpressing factors identified in vascular disease and repair for use in cancer in vitro models	EC factors that contribute to vascular repair (e.g. perlecan, KLF2) also cause anti-cancer effects and those that contribute to vascular disease (e.g. IL-6, endothelin) stimulate cancer, especially on the invasion/metastasis axis
Isolated EC-derived factors will exert specific effects on cancer cells and these effects add nonlinearly in combination	Fractionate (e.g. HPLC) and identify (e.g. MS-MS) EC-secreted products and examine their individual and combined effects on cancer cell behavior	The effects of individual EC-derived products will add nonlinearly, i.e. IL-6 alone stimulates metastasis, but not if other factors from reparative ECs are present.
Modulation of endogenous tumor-associated EC phenotype will affect tumor behavior in animal models	Generate knock-out or knock-in transgenic mice for use in xenograft or spontaneous animals	EC-specific IL-6 knockout (e.g. Tie2-Cre x IL6 LoxP/LoxP) mice will have fewer metastases than normal syngeneic mice.
Tumor ECs exhibit markers of dysfunction	Stain tumor specimens for markers of EC dysfunction; examine in vitro mechanisms of cancer cell or tumor milieu induction of EC dysfunction	Cancer cell or tumor stromal secretion of particular factors (e.g. VEGF, already known to dysregulate EC structure) may correlate with subversion of EC phenotype, increasing with tumor progression.

Drugs that reduce endothelial dysfunction should reduce tumor progression	Use drugs that increase endothelial health (e.g. statins, NSAIDs, p38MAPK inhibitors, or others ID'd based on above experiments) in tumor models	These drugs should inhibit tumor progression. Particular care and control must be taken to parse direct effects on ECs versus nonspecific effects on all cells within tumors.
Activating, pro-inflammatory factors should reduce or reverse EC inhibition of cancer aggressive properties.	Modulate secretions from cancer cells (e.g. overexpress VEGF, TGF-β, cytokines) and dose ECs with cancer conditioned media for assessing effect on EC phenotype.	Factors that activate EC pro-inflammatory signaling should convert the EC phenotype to cancer-stimulatory.

Philosophy, hypotheses, and rationale

This thesis describes the study of endothelial cells (ECs) as plastic paracrine cancer-regulatory cells. The motivation for this work came from established paradigms in vascular repair, where dormant endothelial cells maintain vascular quiescence, physiologically activated endothelial cells stimulate proliferative repair, and dysfunctional endothelial cells are disease-stimulatory. We hypothesized that since endothelial cells line all microvasculature and that all tissues require capillary networks for perfusion, all tissues potentially fall under the regulatory umbrella of endothelial cells. Thus, we posited that reparative endothelial cells inhibit the aggressive aspects of cancer and dysfunctional endothelial cells stimulate these properties. Although this classification scheme may be useful, it is admittedly coarse. There are likely to be many intermediate endothelial cell states, which may manifest as changes in some, but not all, aspects of the cancer-regulatory endothelial phenotype. Furthermore, since our laboratory has in the past used engineered endothelial cell implants in disease-repair models, we borrowed this strategy for use in tumor models. Thus, in this thesis we

asked whether hypotheses and methodologies regarding endothelial roles in vascular injury studies may also apply in the cancer domain.

Examination of the effects of specific endothelial secretions on cancer phenotype

In our studies we found that, similar to vascular disease and repair, EC state directly influenced the behavior of cancer. Reparative ECs inhibited the aggressive aspects of cancer cell behavior and disruption of the quiescent EC state, in a controlled manner by perlecan silencing or by overstimulation with activating molecules, eliminated or reversed the inhibition of metastasis. A common molecule that was induced by both of the EC quiescence-disrupting interventions was IL-6, a pro-inflammatory and pro-metastatic cytokine. Professor Hemann's group at MIT has also identified endothelial IL-6 to be a critical cancer-regulatory secreted factor in its role in forming a protective lymphoma niche after chemotherapy [134]. Moreover, studies over a decade ago identified IL-6 as a melanoma-stimulatory factor [76]. Further examination of the role of endothelial-derived IL-6 in the tumor milieu is warranted, as this molecule is a known stimulator of inflammatory signaling within cancer cells, usually leading to local invasion and distant metastasis [125-126].

Many other molecules that are secreted by ECs have effects in cancer – TGF-β has pleiotropic cancer effects [147], endothelins are associated with breast tumor invasiveness [148-149] and with prostate cancer bone metastasis [150], and CTGF is associated with inhibition of cancer cell proliferation and invasion [151]. Proteomic analysis of EC-released factors may be useful to identify previously unknown cancer-

regulatory molecules emitted from ECs in different states. These studies could follow the example of Furuta et al [146] to identify specific cancer-regulatory roles of biochemically-separated fractions of the endothelial secretome followed by mass spectrometric identification of the components within these fractions. However, there is an important caveat to any work along these lines. Our laboratory has in the past fractionated EC secretions and found nonlinear and cofactor-dependent paracrine effects of the isolates [28], which may make identifying the effects of particular secreted factors challenging. Examination of any specific factors *in vitro* is facile, and the use of matrix-embedded cell constructs in animal tumor models with engineered ECs deficient in or overexpressing any of these molecules is a straightforward extension of the experiments described in this thesis.

However, the ultimate experimental test of any hypothesis regarding a specific endothelial-secreted product would be to specifically modulate its expression in the endogenous endothelium of animals and then use these transgenic animals in tumor growth and/or metastasis models. For example, to examine how the endothelial IL-6 axis affects metastasis, we could generate EC specific conditional knockout animals by crossing commercially available Tie2-Cre mice (C57BL6 background) with in-house generated IL-6 LoxP/LoxP animals and examine metastasis either with xenograft models such as the with the Lewis lung carcinoma (LLC) implantation-resection-metastasis model or by crossing these transgenics with other transgenics to initiate spontaneous tumors (e.g. PyMT-MMTV). We expect that this strategy has a high chance of success, as targeting the second exon of IL-6 has already been successful in

generating viable animals with deleted global IL-6 expression [152], and are already pursuing this idea. We expect tumor-bearing animals with deleted endothelial IL-6 expression to spawn fewer metastases than control mice.

More in-depth examination of intracellular changes within cancer cells effected by ECs.

Another important area of future work will be to more rigorously identify the effects of the intact EC secretome on specific cancer cell signaling networks. There are many potential means to tackle this, but one mechanism which we have begun to explore is the use of cancer cells "addicted" to certain pathways [153-154] to enable a focused but rigorous characterization of the signaling changes induced by exposure to secretions from quiescent ECs.

Towards this end we have already begun to examine the effects of quiescent EC secretions on the behavior of EGFR-addicted non-small cell lung carcinoma cells [155-156]. We have been excited to discover that ECs efficiently target EGFR signaling in EGFR-addicted cell lines and that this occurs in a manner that is fundamentally different that EGFR tyrosine kinase inhibitors (e.g. gefitinib), in that they attack the stability of the structurally abnormal EGFR oncoproteins rather than simply regulating EGFR catalytic activity. We also have been examining EC effects on Wnt/β-catenin signaling in β-catenin-addicted colorectal carcinoma cells. In this context, quiescent ECs again inhibit the necessary critical factors in the target carcinoma cells, but in this setting do so by upregulating an autocrine Wnt signaling inhibitor. Finally, this thesis describes some of

the preliminary efforts made to characterize effects of EC secretions on inflammatory signaling through the canonical NF-κB and STAT3 signaling pathways. Although we derived interesting insights, the described work has just begun to scratch the surface. Future work could utilize, for example, NF-kB addicted These investigations have strengthened the notion that secretions from intact, quiescent ECs are broadly effective at targeting multiple molecular targets in a range of pathologically activated cells.

Another, more generally-applicable methodology for examining cancer-regulatory effects of ECs of various states is to use gene expression profiling to characterize the nature of such effects. Work by Professor Jim Collins et al at Boston University has utilized what they termed "reverse gene interaction network identification" to tune the statistical certainty of gene expression changes identified by cDNA microarray profiling based on the effects of known perturbations to the target cellular networks induced by drugs, biologics, etc. [157-158]. This type of approach would be particularly appropriate for examining the complex effects of sets of endothelial-derived regulatory molecules and is importantly not limited to a particular type of expression profiling (i.e. it would be made to work with mRNA-sequencing gene expression data, proteome profiling data, etc.).

Exploring specific mechanisms of EC phenotypic conversion within the tumor milieu: similarities and differences with dysfunctional ECs.

Our hypothesis that endothelial dysfunction plays a causal role in cancer pathogenesis is intriguing. We recently have initiated a collaboration with Professor

Raphael Bueno (Brigham and Women's Hospital, Boston, MA), a thoracic surgeon who has through his clinical responsibilities acquired hundreds of non-small cell lung carcinoma specimens with matched normal lung tissue. A preliminary analysis of frozen tissue sections that he has graciously provided has already revealed a tremendously-supportive set of data. We developed an algorithm with Will Gibson, a young graduate student in the laboratory, in which small randomly-placed regions of the immunofluorescent images of these tissue sections were analyzed for average intensity of the channel vascular markers (e.g. VE-cadherin) and a quiescence marker (e.g. perlecan) or dysfunction marker (e.g. IL-6) (algorithm description, Fig. 4.1). We found that the endothelium within tumor specimens was dysfunctional in that the average intensity correlation between perlecan and VE-cadherin was insignificant, whereas in matched normal tissues the correlation was strong (Fig. 4.2.). Obvious next steps along these lines will be to probe for other indices of endothelial dysfunction in human frozen tumor specimens by immunofluorescent/immunohistochemical colocalization (e.g. a dysfunction marker with an endothelial marker), qRT-PCR or other gene expression methodology from laser capture microdissected tumor-associated and normal vessels [159], and isolation and short-term culture of bona fide tumor-associated endothelial cells [101, 114].

Another major focus of these studies should be to define the mechanisms by which tumor-associated ECs are coerced in the tumor milieu into a dysfunctional state. This could either be by direct action on the ECs themselves or by pathologic modification of the extracellular matrix layers upon which they reside. The perlecan example mentioned

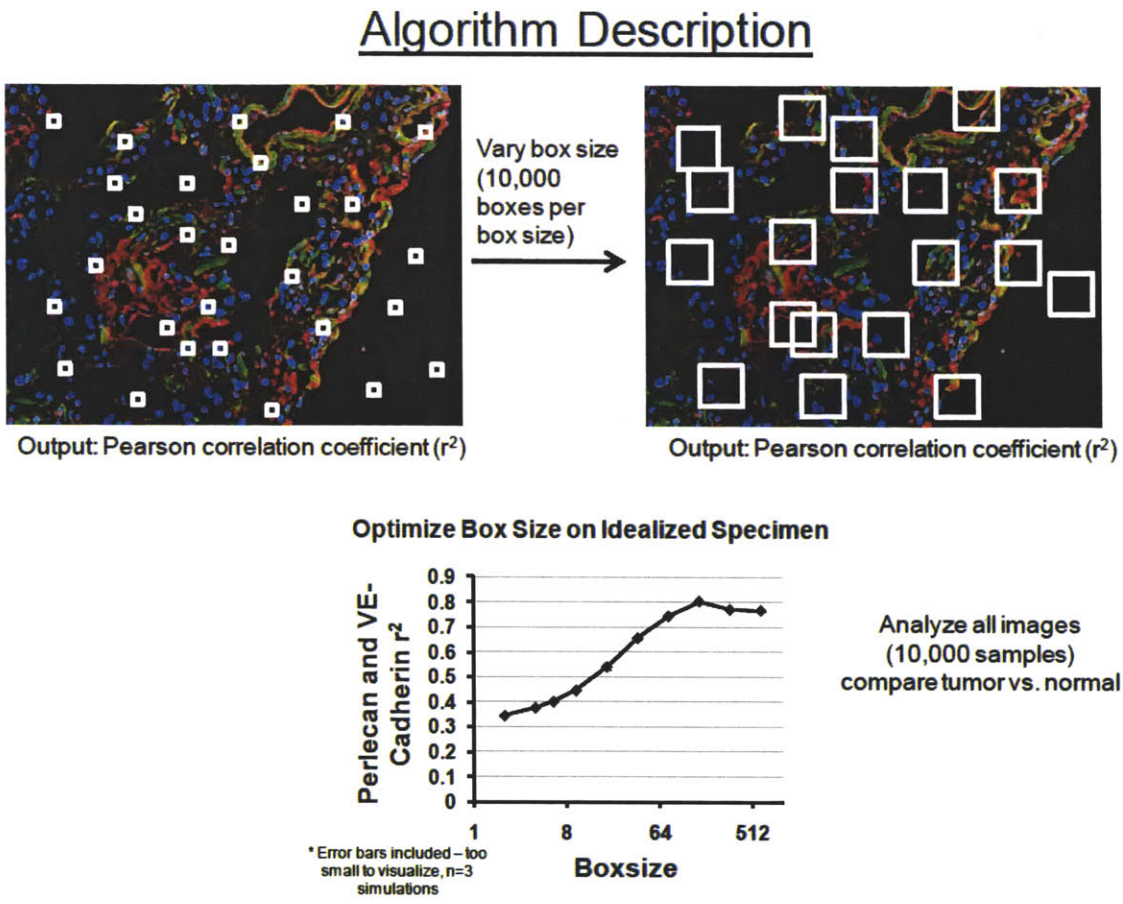

Figure 4.1. Description of the perlecan-VEcadherin colocalization algorithm.

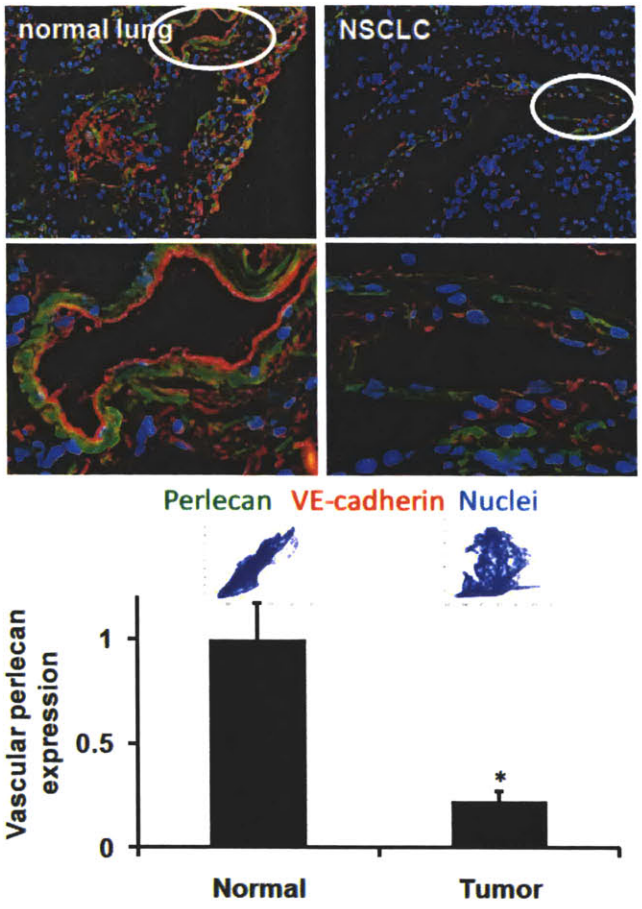

Figure 4.2. Tumor endothelium expresses less perlecan than that of matched normal lung tissue.
Representative immunofluorescent images of a non-small cell lung carcinoma (NSCLC) tumor and matched normal lung tissue stained for perlecan (green) and VE-cadherin (red), with quantification of perlecan-VEcadherin colocalization correlation and sample correlation plots below.

above, in which decreased endothelial perlecan acts as both a biochemical cofactor and an autocrine factor to modify the endothelial phenotype, is an intriguing example of this paradigm. Other EC-expressed proteoglycans such as versican [160] have already been identified as key molecular mediators in cancer. Other obvious inciting factors could be pathologic concentrations of known pro-inflammatory (e.g. TNF-α, IL-6 [122]) and pro-angiogenic factors (e.g. VEGF [67]). Finally, we have been able to identify NF-kB activation within the endothelial compartment of tumors (Fig. 4.3). This phenomenon is heterogeneous but present within many human non-small cell lung carcinoma specimens. We predict that this phenomenon is widespread, given the known importance of inflammation within the tumor milieu.

An increasingly appreciated aspect of intracellular communication is the microvesicular route [161-163]. It is noteworthy that not all factors that cause endothelial dysfunction within a tumor necessarily derive from the tumor itself or even the host. Genotoxic chemotherapy, for example, induces IL-6 expression in ECs [134] and could therefore be a major driver of endothelial dysfunction and cancer stimulation or protection. Here, again, a reverse network engineering approach [157] may be useful in the careful identification of EC signaling networks by gene expression analysis that may go awry due to the stresses placed on these cells within the tumor milieu.

These issues are likely dynamic and change with tumor progression. Less aggressive tumors may be more controllable by quiescent ECs but as additional mutations are accrued by cancer cells, the more apt they may be to coopt stroma to

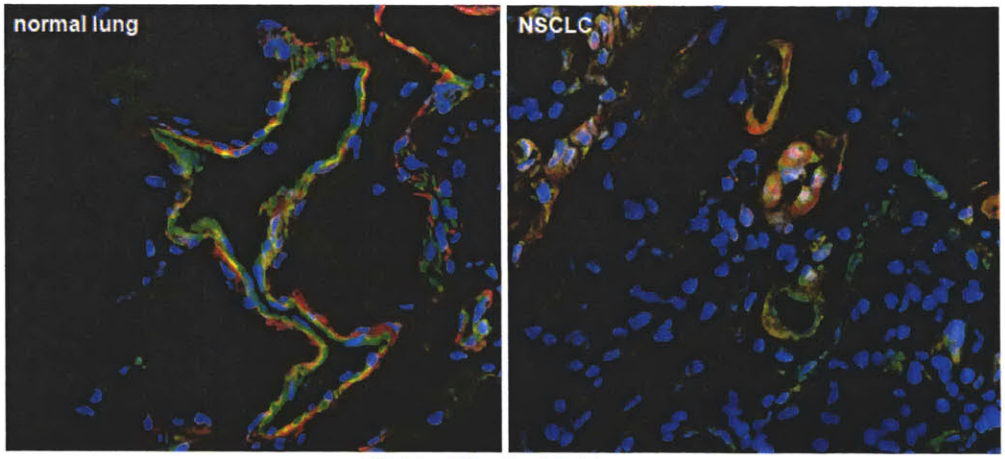

Figure 4.3. Tumor endothelium displays heterogenous NF-κB activation. Compared to normal lung vessels, which contain large amounts of latent cytosolic NF-κB, tumor endothelium has disordered structure and contains foci of NF-κB activation (NF-κB p65 nuclear localization).

facilitate further tumor progression. For example, metastatic melanoma cells can more fully alter the gene expression patterns of fibroblasts in a manner that then makes the fibroblasts more melanoma-stimulatory [164].

Finally, even the literal identity of ECs within tumors may change. Ragu Kalluri and colleagues have elegantly demonstrated an endothelial origin of a large fraction of carcinoma-associated fibroblasts [85], illustrating the possibility of the endothelial-to-mesenchymal transition as a contributing factor for modulation of EC paracrine regulatory properties. Studying these issues will require careful analysis and sophisticated animal models in which any cell that is or was endothelial is permanently marked.

Translational implications: development of novel pharmacologic and cell-based cancer therapies.

The ultimate goal of all of the preceding proposed work is to develop more effective cancer therapies. A quickly-testable class of therapeutic candidates may be based on matrix-embedded endothelial cells. Such endothelial cell-based therapies have already been deemed to be safe and effective in clinical trials for vascular repair. We have licensed a patent to Pervasis Therapeutics (Cambridge, MA) to explore these possibilities in greater depth, and thus far preclinical studies have been very promising. They hope to start clinical trials in humans in the near future.

Another class of therapy that may emerge from these expanded therapies is a pharmacologic intervention designed to reverse endothelial dysfunction within tumors.

Such a drug, if safe, would effectively turn a tumor's endogenous ECs against it. There are already seem to be promising candidates. HMG-CoA reductase inhibitors ("statin" drugs) and NSAIDs both inhibit inflammatory signaling within many target cell types, including ECs, and are associated with reduced cancer mortality [95]. However, these studies are purely correlative and cannot rule out direct effects on the cancer cells themselves or other stromal elements. These issues deserve more attention.

Conclusion

Although it has been considered in the past, inclusion of ECs as part of the cancer-regulating stroma is still an emerging field, and there exist many opportunities to make a significant research impact along many axes. I hope that our laboratory's in-depth knowledge regarding the biology of vascular repair will continue to motivate novel insights regarding EC-cancer paracrine interactions, with the ultimate goal of translating these insights into more effective cancer diagnostics and therapies.

CHAPTER 5. REFERENCES

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APPENDIX A. RESULTS OF ADDITIONAL EXPERIMENTS NOT INCLUDED IN MAJOR CHAPTERS

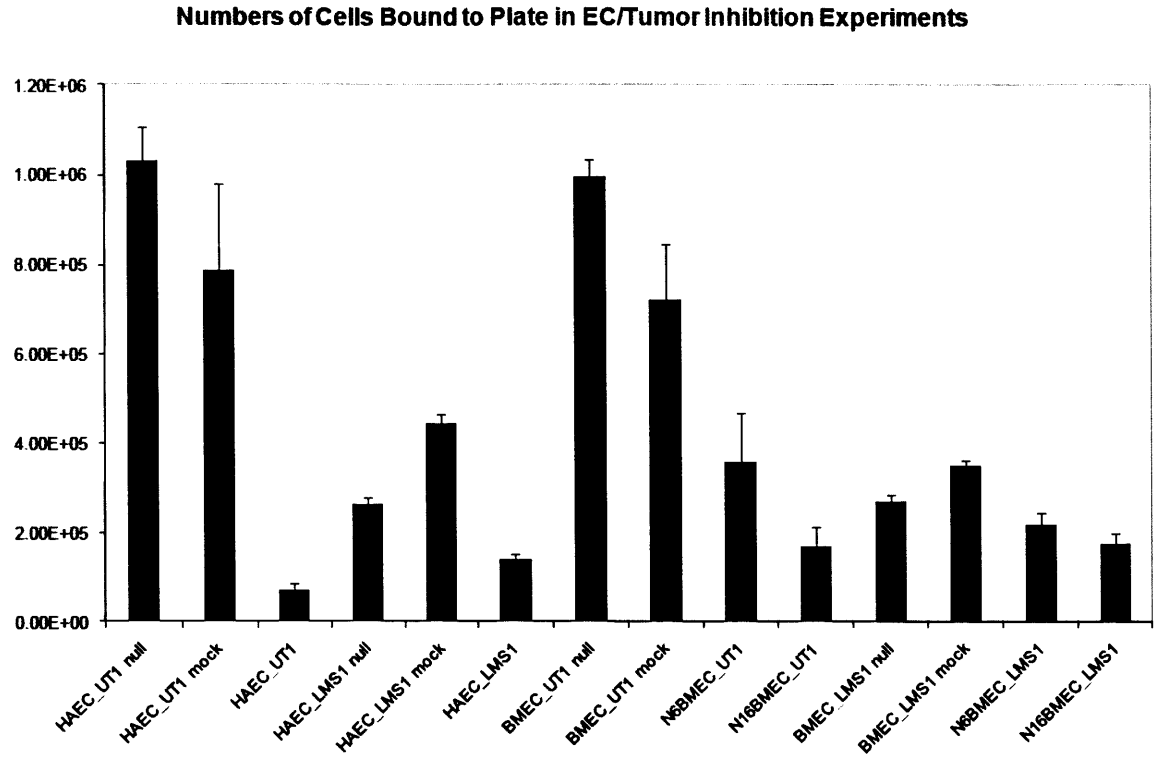


Figure A.1. Leiomyosarcoma cell proliferation is inhibited by the presence of matrix-embedded ECs.

The numbers of leiomyosarcoma cells attached to each well of a 6-well plate with floating matrix-embedded ECs is shown here after 6 days of coculture (5,000 cancer cells seeded per well, 50,000 ECs seeded per Gelfoam sponge). Error bars are standard deviations. HAECs = human aortic endothelial cells. BMEC = bone marrow endothelial cells. UT-1 = SK-UT-1 leiomyosarcoma cells. LMS-1 = SK-LMS-1 leiomyosarcoma cells. N.B. there were no Transwell barriers separating the cancer cells from the ECs.

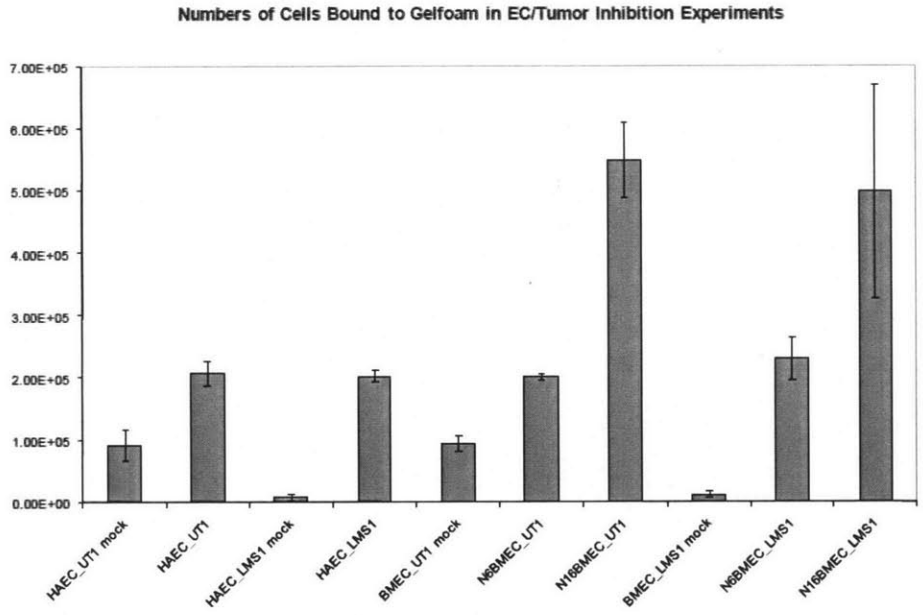


Figure A.2. Relatively few ECs are required to inhibit the proliferation of leiomyosarcoma cells.

Number of cells released from Gelfoam matrices corresponding to the experiment from Figure A.1.

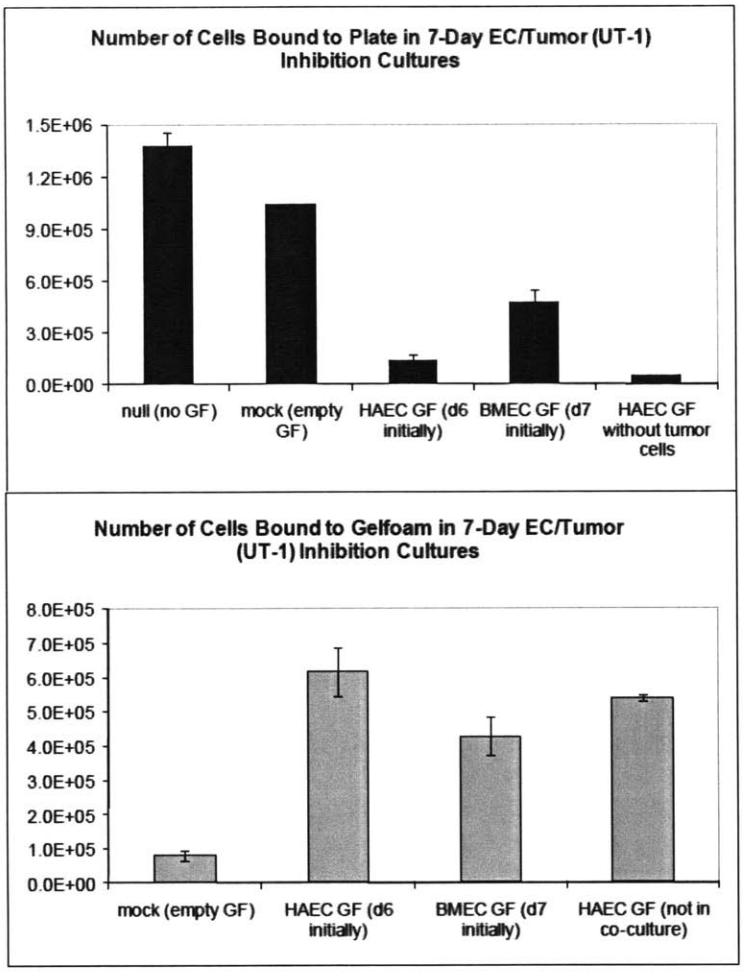
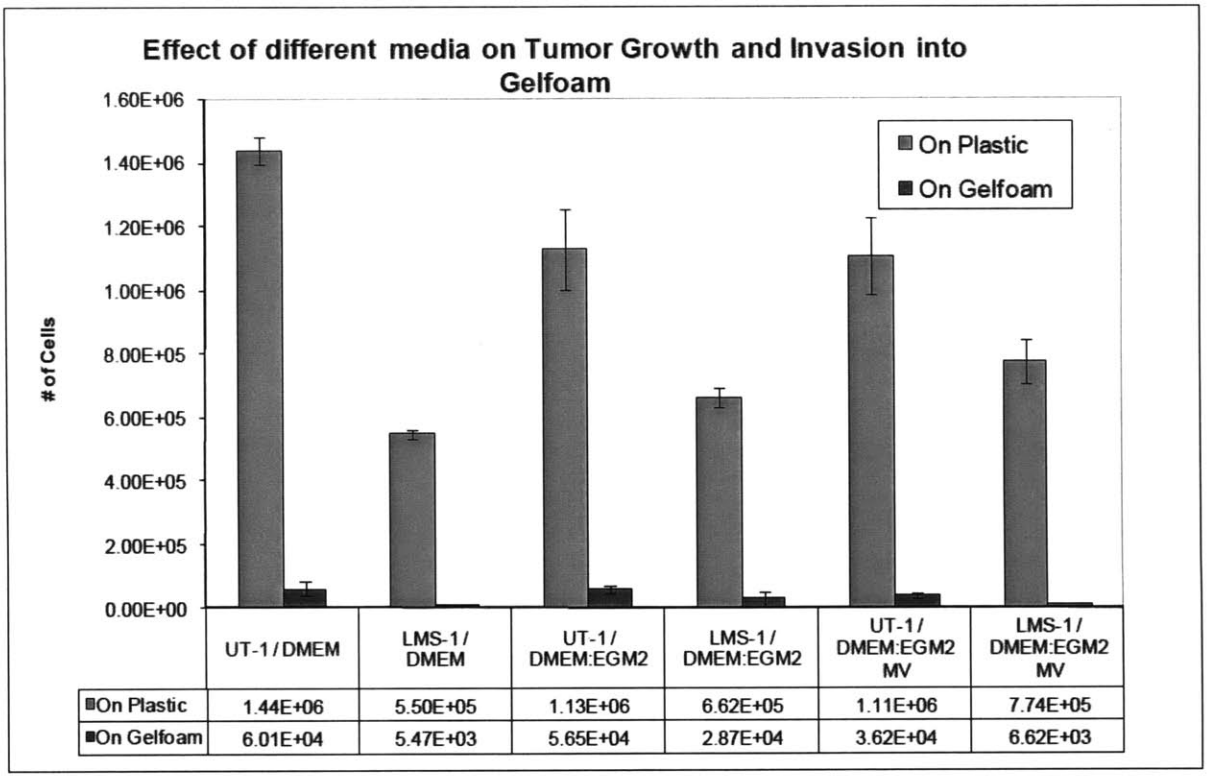


Figure A.3. When more "confluent" matrix-embedded ECs are used, the inhibition of SK-UT-1 cancer cell number is greater (dose-dependent paracrine effect?), with little gross difference between EC type when normalizing per cell.

The number of cells per well (6-well plates) of SK-UT-1 cells after 7-day cocultures (no Transwell barrier separation) with matrix-embedded HAECs and BMECs is shown.



	UT-1 / DMEM	LMS-1 / DMEM	UT-1 / DMEM:EGM2	LMS-1 / DMEM:EGM2	UT-1 / DMEM:EGM2 MV	LMS-1 / DMEM:EGM2 MV
On Plastic	1.44E+06	5.50E+05	1.13E+06	6.62E+05	1.11E+06	7.74E+05
On Gelfoam	6.01E+04	5.47E+03	5.65E+04	2.87E+04	3.62E+04	6.62E+03

Figure A.4. Adherent cancer cells do not detach and colonize acellular Gelfoam sponges in great number.

The number of cancer cells per Gelfoam sponge piece after 7 days of culture with a floating acellular sponge above the cancer cell layer.

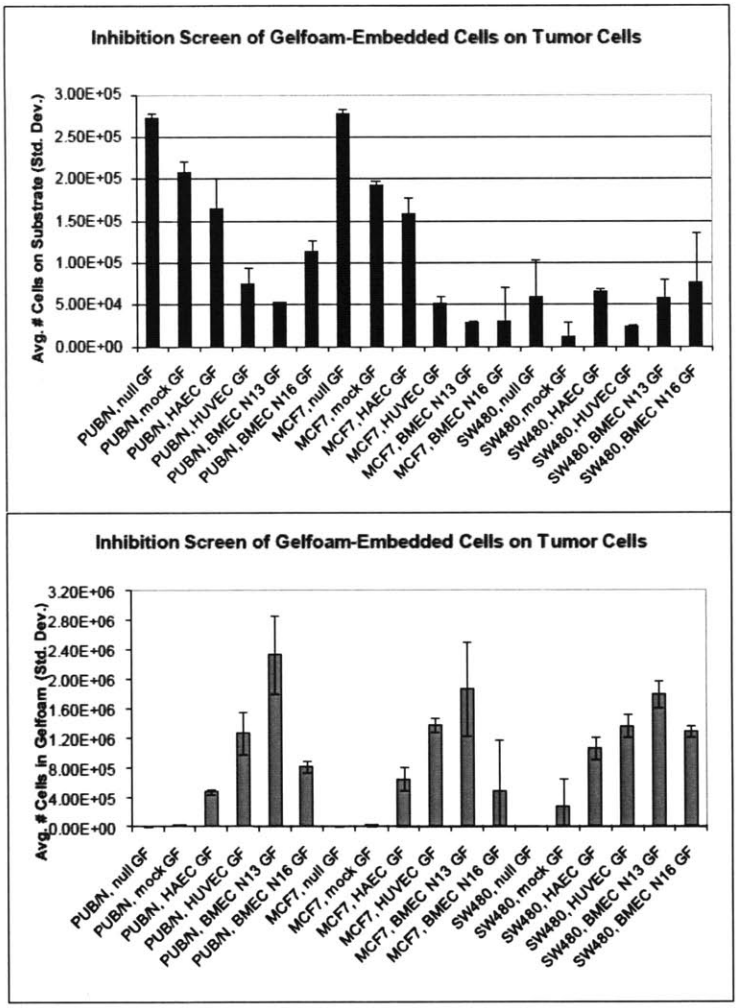


Figure A.5. Coculture with matrix-embedded ECs inhibits the proliferation of lung, breast, and colorectal carcinoma cells.

7-day coculture experiment without Transwell barrier separation of PUB/N lung carcinoma, MCF7 breast carcinoma, and SW480 colorectal carcinoma cells from HAECs, HUVECs, and BMECs.

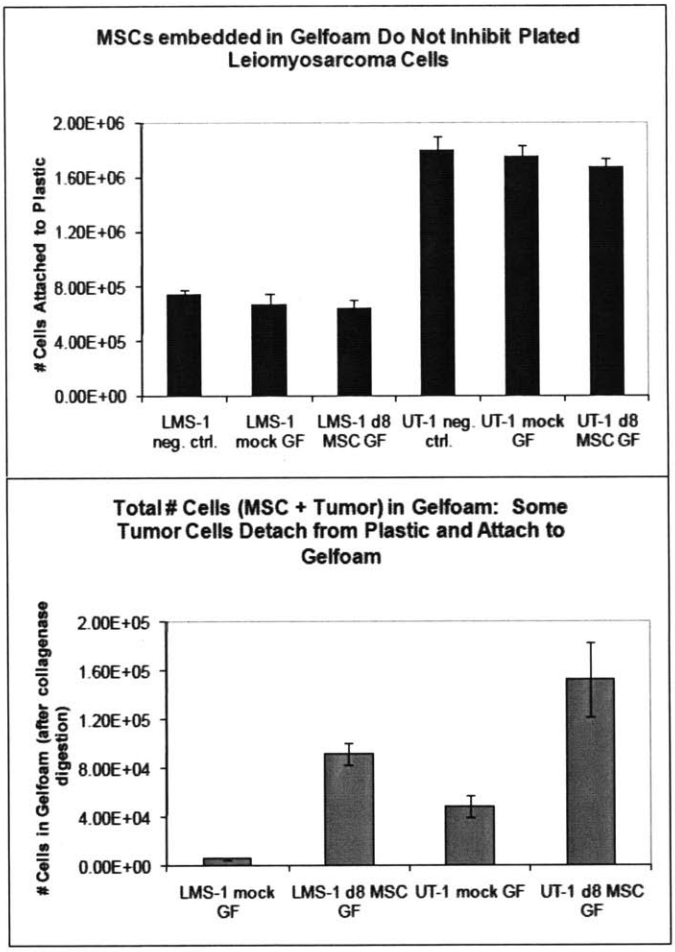


Figure A.6. SK-LMS-1 and SK-UT-1 proliferation are not inhibited by small numbers of embedded mesenchymal stem cells (MSCs).

7-day coculture experiment with embedded MSCs and adherent cancer cells.

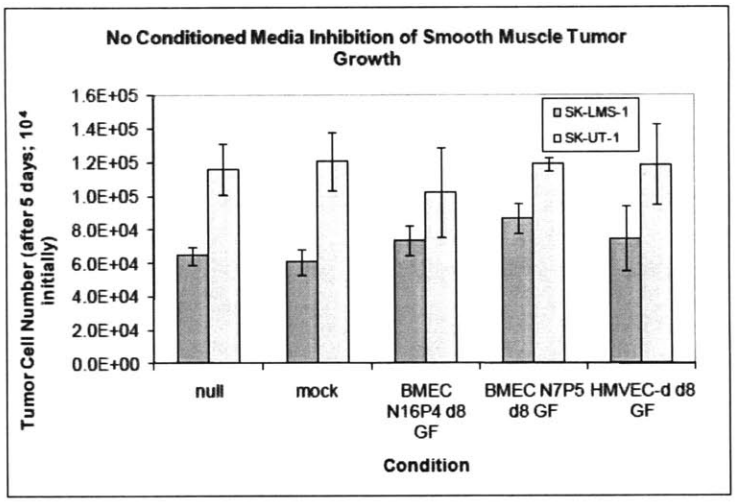


Figure A.7. Conditioned medium from BMECs and HMVECs do not inhibit well the proliferation of SK-LMS-1 or SK-UT-1 cells, 5 day culture experiment.

Numbers of adherent cancer cells after 5 days (10,000 seeded initially) of coculture with matrix-embedded BMECs and HMVECs (dermal microvascular ECs).

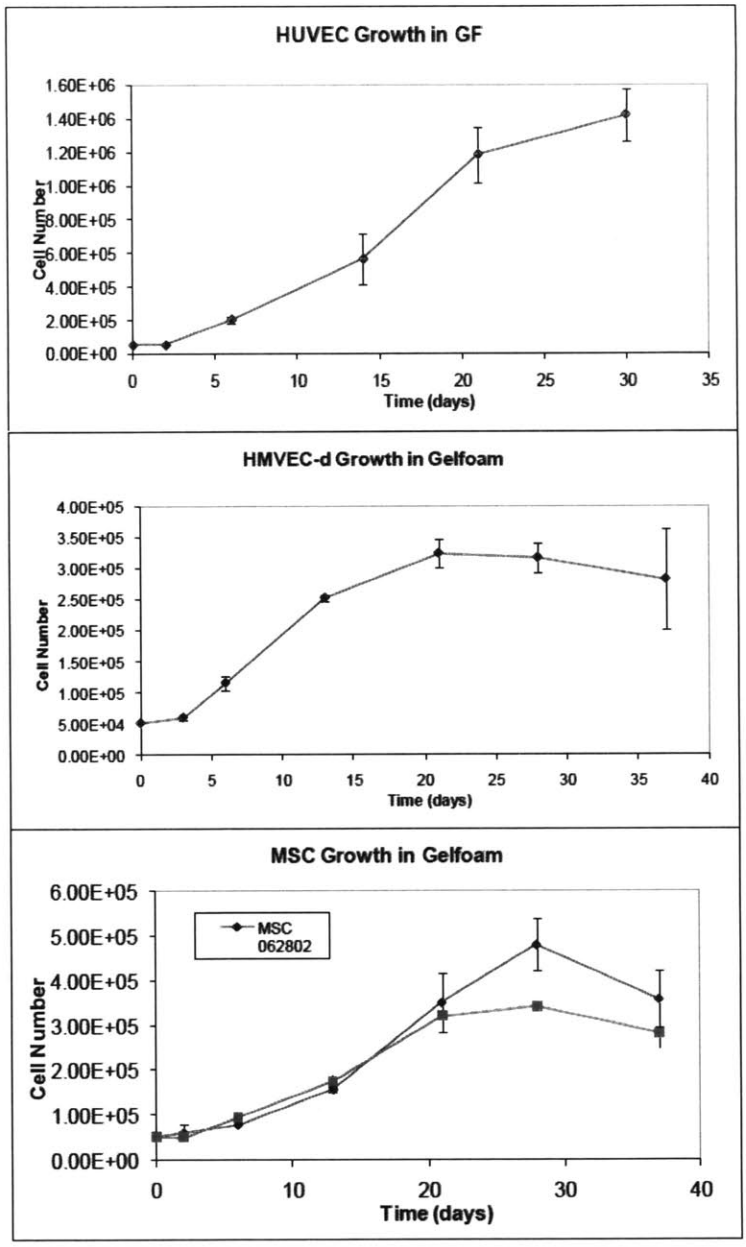


Figure A.8. Growth kinetics of MSCs, HUVECs, and HMVECs in Gelfoam sponges.

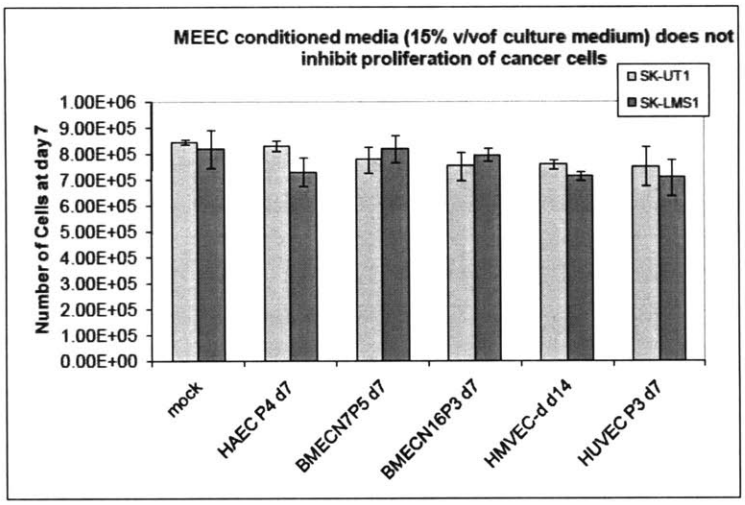


Figure A.9. Replacing 15% v/v of the cancer cell culture medium with MEEC-conditioned medium does not inhibit proliferation.

7-day culture of SK-UT-1 and SK-LMS-1 cancer cells with 15% v/v MEEC conditioned medium.

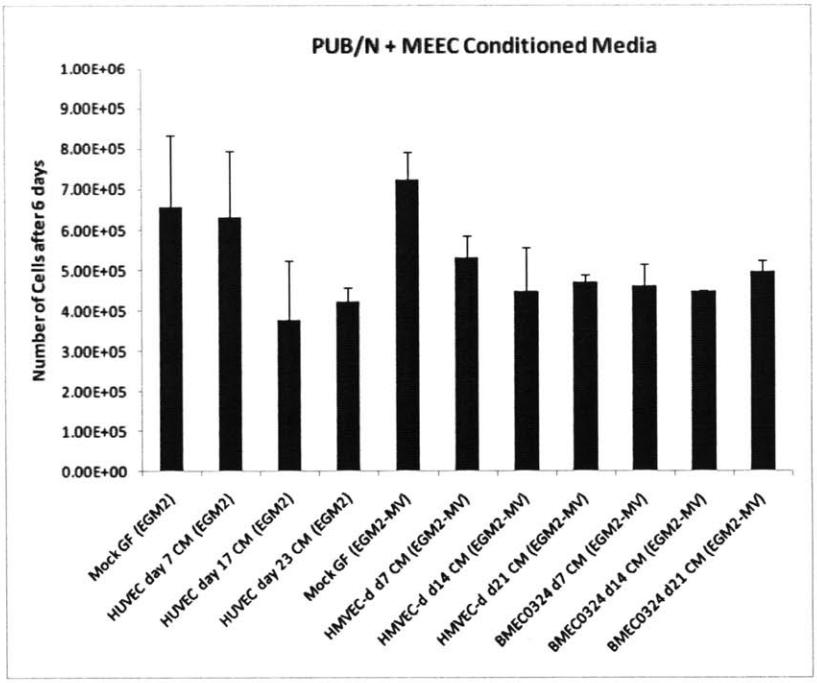


Figure A.10. Replacing 10% of the culture medium with 5X-conentrated MEEC-conditioned medium can inhibit proliferation of PUB/N cells.

6-day culture of PUB/N lung carcinoma cells (1,000 cells seeded per well of 12-well plate). 10% of the culture volume was replaced with 5X-concentrated MEEC-conditioned medium (either from HUVECs, HMVECs, or BMECs).

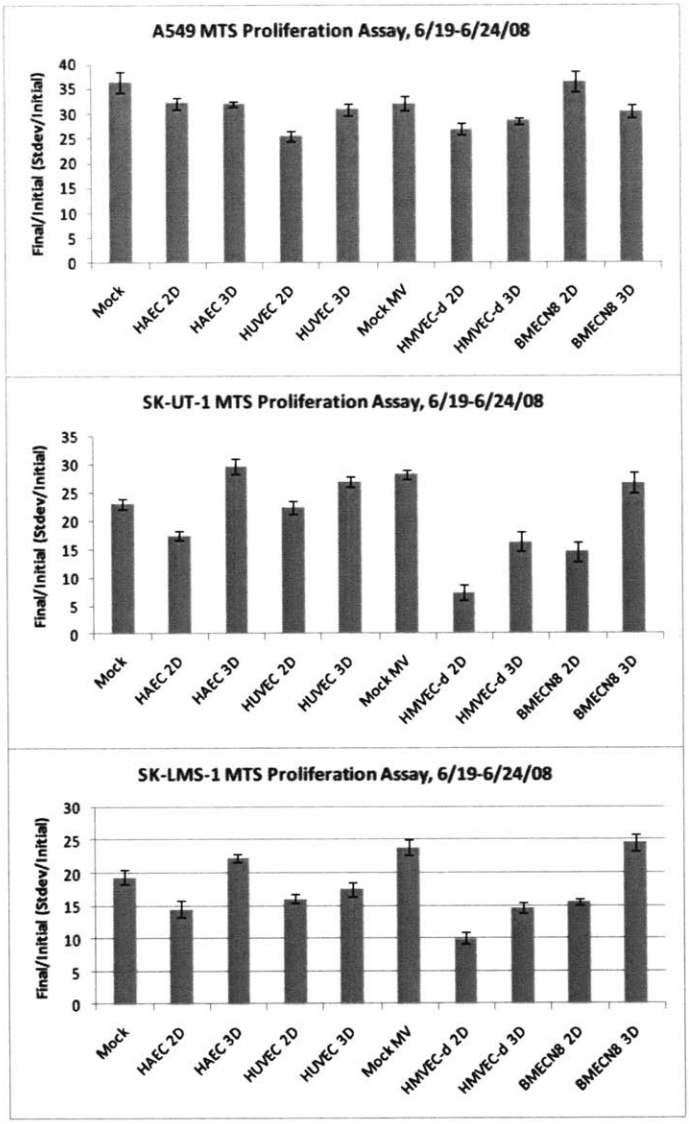


Figure A.11. Proliferation (MTT) of A549 lung carcinoma, and SK-UT-1 and SK-LMS-1 leiomyosarcoma cells with CM from HAECs, HUVECs, HMVECs, and BMECs grown on gelatin-coated plates (2D) or within Gelfoam matrices (3D).

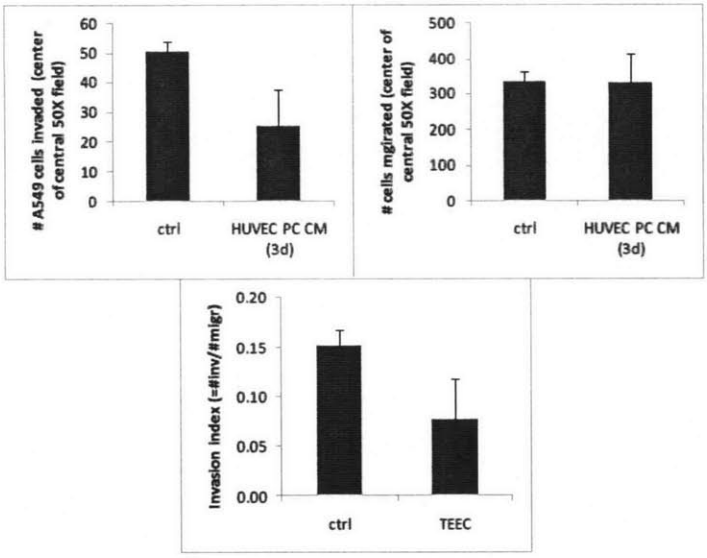


Figure A.12. Media conditioned by matrix-embedded HUVECs inhibits the in vitro invasiveness, but not migration, of A549 cells.

A549 cells were cultured for 4 days in CM, then harvested and seeded equal numbers into chemoinvasion/migration chambers (BD).

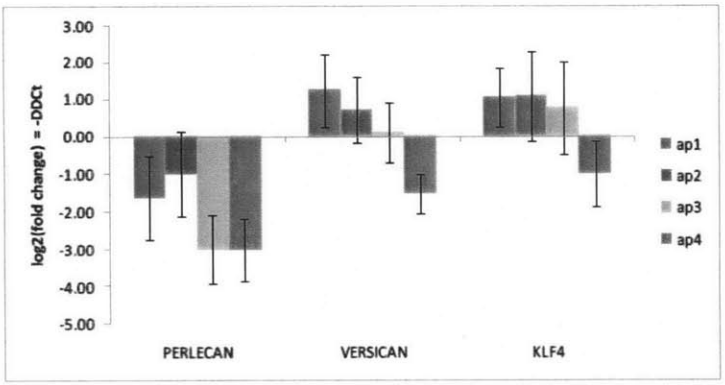


Figure A.13. Perlecan silencing in HUVECs is uncorrelated to effevts on other selected endothelial genes.

4 different lentiviral plasmids were transduced into HUVECs and expression of perlecan, versican, and KLF4 was examined by qRT-PCR.

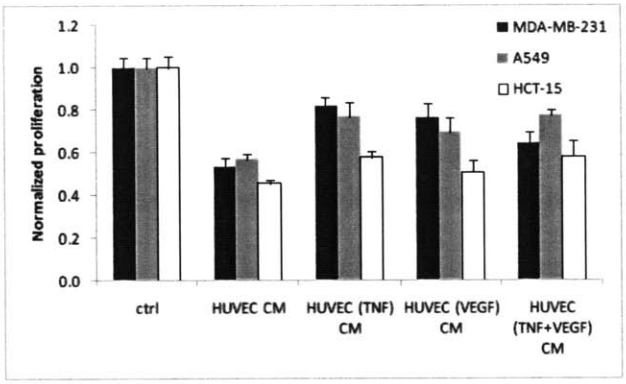


Figure A.14. Proliferation of A549 lung, MDA-MB-231 breast, and HCT-15 colorectal carcinoma cells in media conditioned by ECs, inflamed ECs, VEGF-stimulated ECs, and TNF+VEGF-stimulated ECs.

Conditioned media (48 hours, 8 L MCDB-10 per 10-cm dish of conlfluent ECs) was collected AFTER the EC activation.

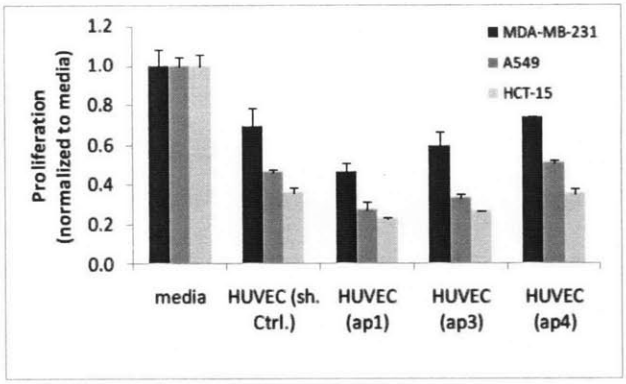


Figure A.15. Perlecan silencing in ECs does not change dramatically how they inhibit the proliferation of MDA-MB-231, A549, and HCT-15 cells.

These cancer lines were cultured for 4 days in media conditioned by null-modified or perlecan-silenced HUVECs.

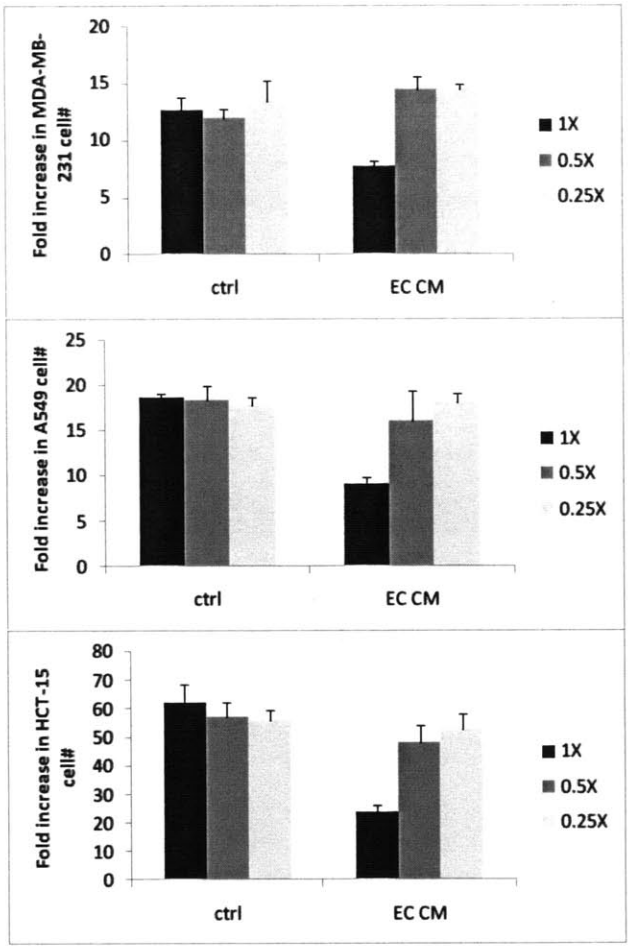


Figure A.16. Diluted EC media cannot effectively inhibit A549 lung, MDA-MB-231 breast, or HCT-15 colorectal carcinoma proliferation.

Media conditioned by postconfluent HUVECs (48 hours, 8 mL MCDB-10%FBS per 10-cm dish) was either used or diluted and used for 4-day cultures of the cancer cell lines.

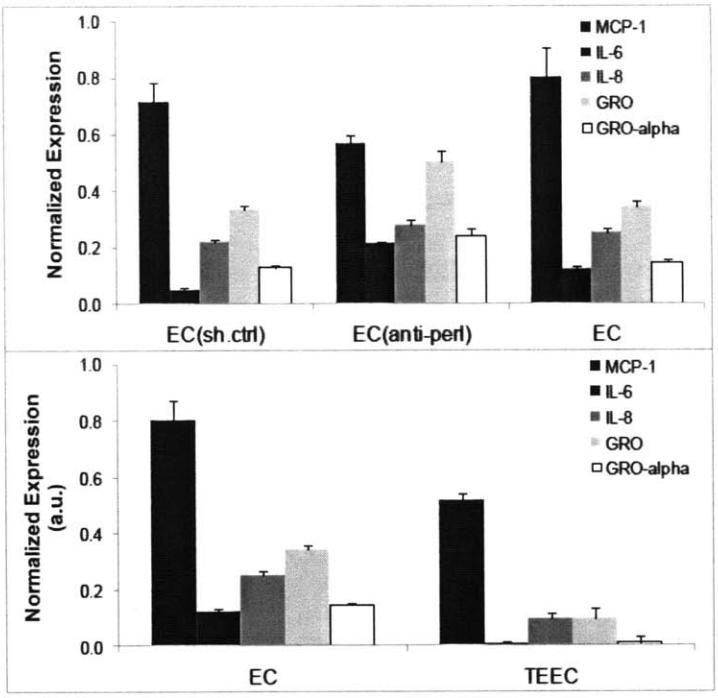


Figure A.17. Perlecan silencing increases the secretion of several cytokines from ECs grown on gelatin-coated plates, but matrix-embedding reduces cytokine output.

Values are normalized to the positive control spots. TEEC = tissue-engineered EC, which is the same as matrix-embedded EC.

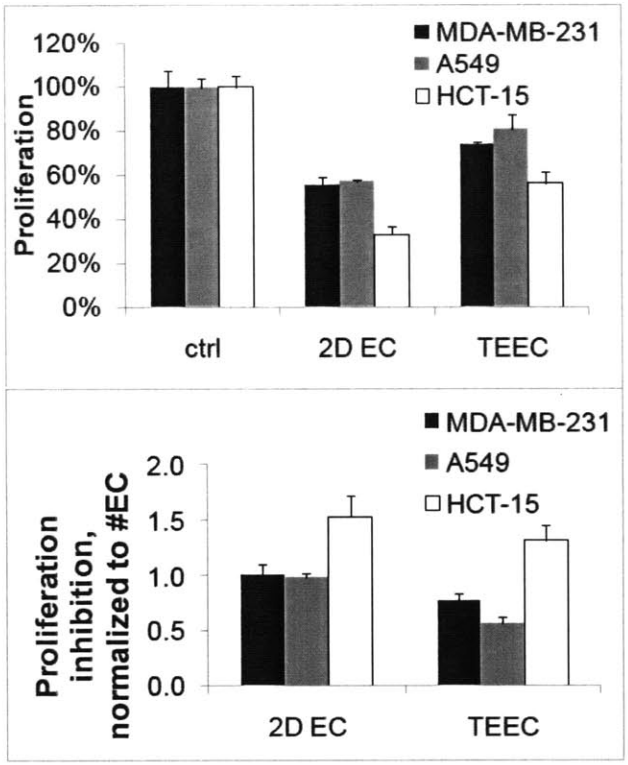


Figure A.18. Matrix-embedded ECs inhibit the proliferation of MDA-MB-231 breast, A549 lung, and HCT-15 carcinoma cells similarly, when percent inhibition is normalized per endothelial cell.

TEEC = tissue-engineered EC, which is the same as matrix-embedded EC.

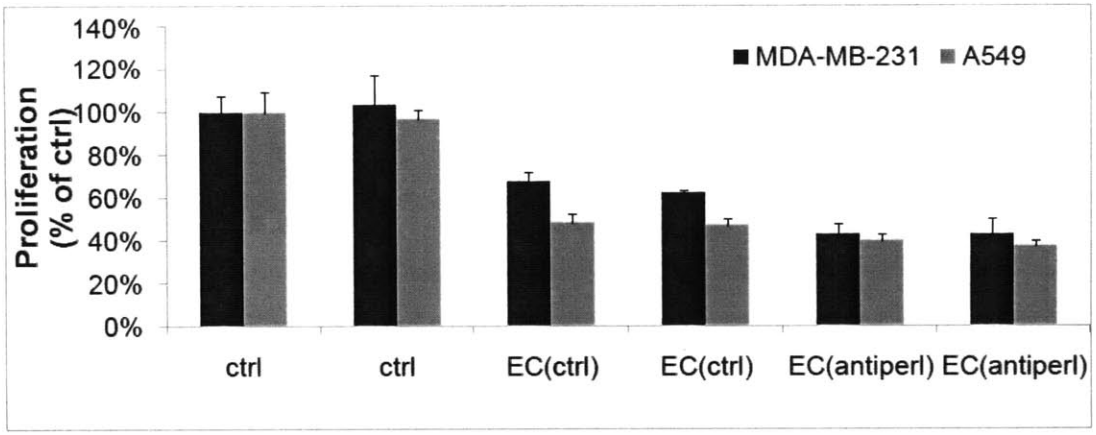


Figure A.19. IL-6 neutralization has no effect on proliferation of MDA-MB-231 breast or A549 lung carcinoma cells.

1 ug/mL goat polyclonal IgG or 1 ug/mL IL-6 neutralizing antibody (RnD systems) were added to the culture medium > 30 minutes before use in cell culture.

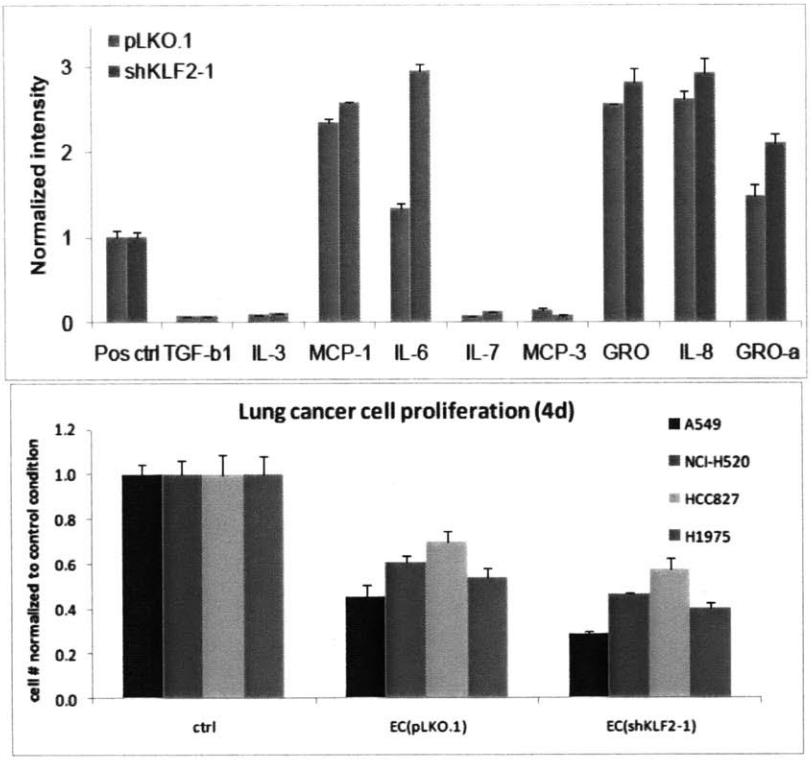


Figure A.20. EC transduction with shRNA targeting KLF2 upregulates IL-6 and possibly GRO-α/CXCL1, which could indicate a pro-inflammatory/metastatic EC regulatory phenotype, and may inhibit net growth of cancer cells.

KLF2 is a vasoprotective gene upregulate by healthy shear stress and HMG-CoA reductase inhibitors and downregulated in atherosclerotic lesions. If the tumor milieu causes KLF2 reduction it could therefore help to make ECs dysfunctional and possibly metastasis-stimulatory. The upper results show the intensity of a cytokine dot blot of conditioned media (48-hrs, 8 mL MCDB-10%FBS per plate). The lower results show the results of MTT data after a 4-day culture in EC-conditioned media. pLKO.1 denotes transduction with the empty pLKO.1 plasmid (i.e. plasmid without shRNA targeting KLF2).

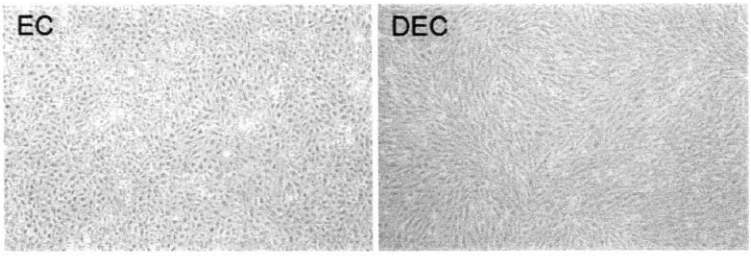


Figure A.21. DEC morphology is spindly, larger, and elongated relative to the cobblestone morphology of postconfluent ECs. Phase contrast image (4X objective).

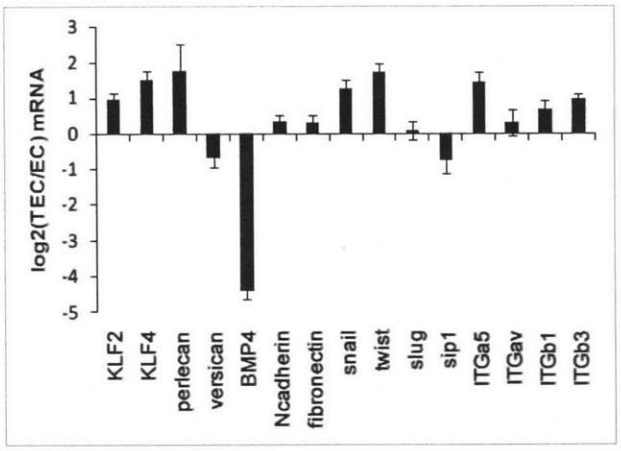


Figure A.22. DEC (in vitro dysfunctional EC) gene expression show increased KLF2/4 (possible compensation?), downregulation of BMP4, upregulation of Snail and Twist, and upregulation of integrin $\alpha 5\beta 3$. qRT-PCR data after 4 days of culture.