Beta 3 Integrins:

Negative Regulators of Angiogenesis

by

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ABSTRACT

A method was developed to isolate and purify primary murine endothelial cells from lung tissue (MLEC). The cells generated by this method were characterized by immuno-fluorescence detection and FACS analysis and expressed specific antigens including PECAM-1, ICAM-1, ICAM-2, VCAM-1 and VE-cadherin. Using this method, cells from wild-type and beta 3-integrin-deficient animals were purified and used to determine the specificity of a novel potential anti-angiogenic drug.

This study shows that tumstatin, a fragment of the alpha 3 chain of collagen IV, inhibits proliferation, inhibits total protein synthesis and specifically inhibits CAP-dependent protein synthesis in MLEC. These effects do not occur when beta 3-null MLEC are treated with tumstatin or any of its derivatives. Nor do they occur in mouse embryonic fibroblasts which do express beta 3 integrin. The inhibition by tumstatin also occurs in *in vivo* angiogenesis assayed using a Matrigel plug insert. Similarly to *in vitro* assays, tumstatin failed to inhibit angiogenesis in beta 3 integrin-deficient animals. These results suggest that $\alpha v\beta 3$ integrin is necessary but not sufficient for the activity of tumstatin. Further studies are required to identify $\alpha v\beta 3$ integrin-associated factors in endothelial cells which determine tumstatin's endothelial cell specificity.

Matrigel plug assays were also used to demonstrate that the loss of beta-3 integrin enhanced VEGF-induced angiogenesis. Results also show that VEGF-induced angiogenesis was enhanced in aortic ring explants from beta 3-null animals.

These data suggest a new role for beta 3 integrin as a negative regulator of angiogenesis, both as a receptor for an endogenous inhibitory molecule and as an inhibitor of VEGF-induced angiogenesis.

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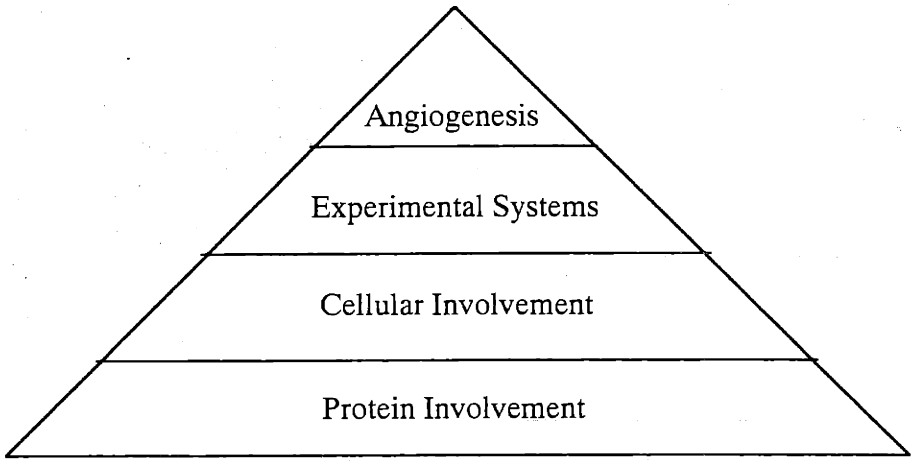
Chapter 1: Thesis Introduction



In this chapter I will introduce the overall process which I have investigated, discuss how one studies it and then outline the cell types involved and the molecules expressed in those cells relevant to the discussions which will follow.

Vascular Development

Angiogenesis

The development of mature vasculature occurs in a biphasic process. The vasculature to a naïve observer appears to be one of the most simple yet important organs. Its generation in the developing embyo occurs first by the process of **vasculogenesis**, the differentiation of endothelial cells from progenitor cell types and their assembly into tubes, and is followed by **angiogenesis**, the formation of new vessels from this (or any) pre-existing network (Risau, 1991a). The early differentiation of

progenitor cells results in an accumulation of angioblasts in the yolk sac (Noden, 1991; Risau, 1991c). Angioblasts coalesce into a collection of blood islands in the embryo and the fusion of such blood islands forms the primary vascular plexus, the most simple network of tubes.

Angiogenesis proceeds from this initial tubular network, expanding the endothelial population and sprouting to form new vessels. The process is characterized by high levels of endothelial cell proliferation, migration and "differentiation or specialization", as well as recruitment of supporting cells of the vasculature including specialized smooth muscle cells, pericytes, and astrocytes. Both vasculogenesis and angiogenesis are stimulated by a host of growth factors including vascular endothelial growth factor (VEGF), which will be discussed at length elsewhere in this document. Existing vessels sprout to originate new vessels. Vessels become more complex by splitting, through intussusceptions, and by fusing, through anastomosis, to form a complete circuitry that supplies oxygen and nutrients to all organs and tissues and draws waste from them.

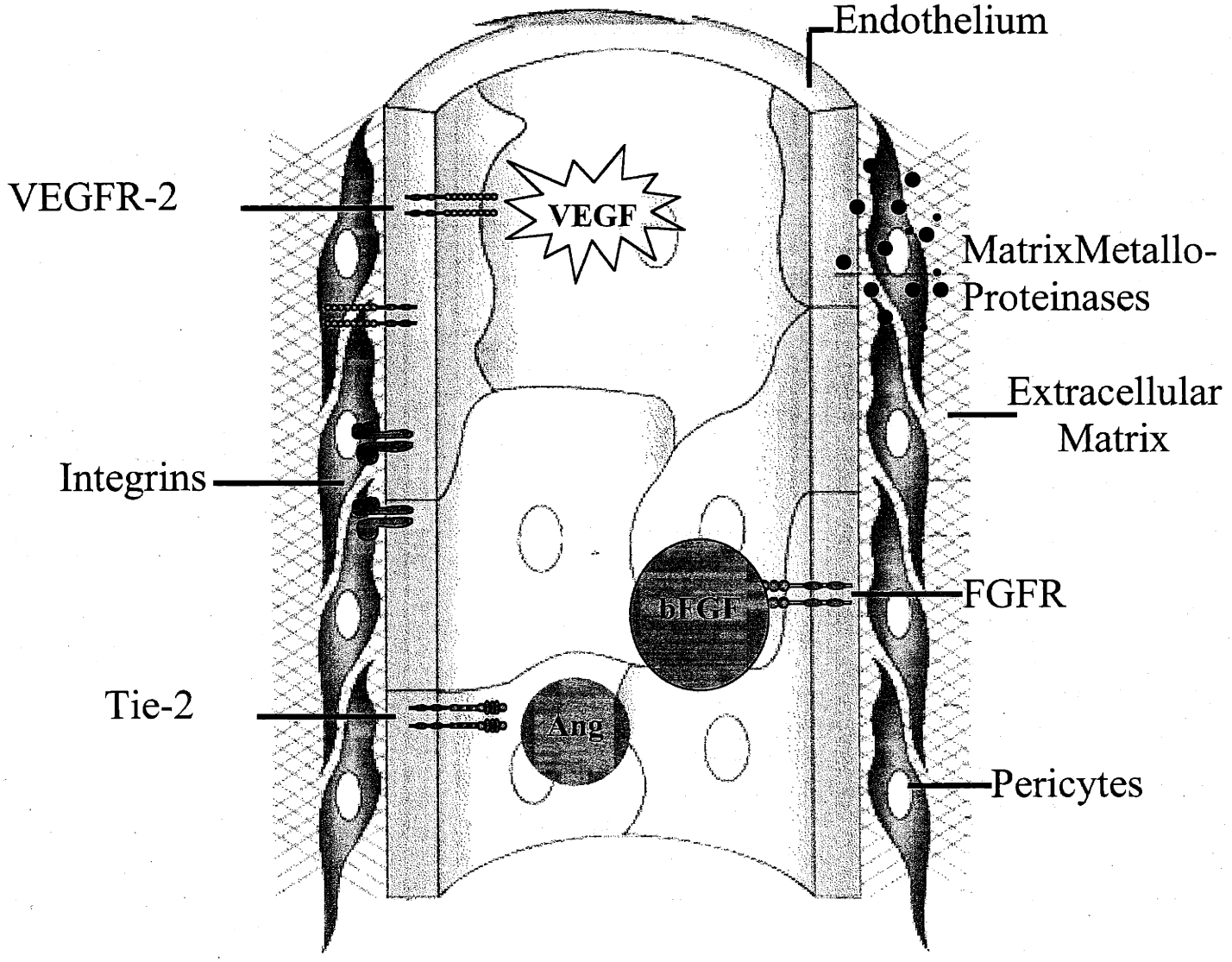


Figure 1.1: Schematic Drawing of Angiogenic Vessel

A cross section of a vessel is shown. Lumenal to ablumenal organization of endothelium, pericytes and matrix are included. Vascular integrins, matrix, MMPs, growth factors and receptors will be discussed in the following pages (adapted from Ruoslahti, 2002).

Normal angiogenesis in the adult is limited to highly ordered processes in the female reproductive cycles (ovulation, menstruation, implantation and pregnancy) (Fraser and Lunn, 2000; Redmer and Reynolds, 1996; Weston and Rogers, 2000) and wound healing (Kurdy et al., 1996; Nissen et al., 1998; Tonnesen et al., 2000). Unfortunately angiogenesis in the adult also contributes to the pathogenesis of a variety of conditions (diabetic retinopathy, rheumatoid arthritis, psoriasis and tumor angiogenesis) (reviewed in Folkman, 1995)

As with embryonic development, adult angiogenesis depends on the successful activation of endothelium yet is also dependent on its return to quiescence. In the absence of this control, angiogenesis will contribute to pathological conditions. The hallmark of proliferative diabetic retinopathy (PDR) is abnormal angiogenesis that may ultimately cause severe vitreous cavity bleeding and/or retinal detachment (Danis et al., 2001). The synovium of patients with rheumatoid arthritis exhibits active angiogenesis accompanied by high density of microvessels with active VEGF/receptor complex contributing to the pathogenesis of this disease (Giatromanolaki et al., 2001).

Although not causative in the inception of cancer, the development of new blood vessels from pre-existing ones is required for tumor growth and metastasis (Bicknell and Harris, 1996; Folkman and Hanahan, 1991; Zetter, 1980). The successful expansion of the tumor cell population and the coordinated endothelial cell expansion are facilitated by an "angiogenic switch", generating a predominantly pro-angiogenic stimulus. The concept of a switch was introduced in the early 1990s by Folkman and colleagues and inspired a new perspective in tumor biology research. In the absence of such a switch the tumor is unable to survive and grow larger than a few mm^3 (Folkman and Hanahan,

1991). Tumor cells retain the ability to divide but in the absence of sufficient blood supply, cells distant from vessels lack proper nutrient supply, oxygenation and the ability to remove cellular waste. These areas devoid or deficient in such processes will fail to survive, the cells will necrose, and the tumor will no longer continue to expand. The key events leading to the turning of the switch however are still not clearly elucidated.

Not all angiogenic pathologies are due to an overactive vascular system; insufficient activation of angiogenesis also creates pathological conditions leading to insufficient oxygen supply, inflammation and cell death. Conditions including restenosis, thrombosis, hypertension, atherosclerosis, peripheral vascular disease in congestive heart failure, and ischemia, either chronic or angioplasty- or stent-induced, are all confounded by defects in vasculature (McKay and Gaballa, 2001). The insufficient growth of collateral vessels causes a major problem in atherosclerotic cardiovascular disease (Partanen and Paavonen, 2001). A reduced density of arterioles and capillaries in various microvascular beds is an important characteristic in many forms of hypertension (Boudier, 1999). Osteoarthritis, in contrast with rheumatoid arthritis discussed above, appears to be a condition developing from the failure of appropriate signaling through the VEGF receptor resulting in activation of the p53 pathway and degeneration of the synovium (Giatromanolaki et al., 2001). Chronic limb ischemia likewise results from the inability to induce the VEGF stimulation needed for vascular proliferation (Levy, 1999). Controlled stimulation of angiogenesis would be of therapeutic value in all of these conditions.

Exploration of these pathological conditions provides insight into the normal molecular mechanisms of angiogenesis. In addition, these two classes of diseases, the

first where the induction of therapeutic angiogenesis can repair the tissue damages (arteriosclerosis, myocardial infarction, limb ischemia) and the second where inhibition of pathological angiogenesis can cure the disease or delay its progression (retinopathies, benign and malignant angiogenic tumors, progression of malignant tumors), jointly drive the development of angiogenesis-specific treatments of complex disease (Timar et al., 2001). A complete understanding of angiogenesis through investigation of these pathologies, as well as identification of specific inhibitors and activators, therefore has great potential in rational choices in treatment of many pathological conditions with defects in vascularity.

At the cellular level, angiogenesis is a coordinated process of the proliferation, migration, and interaction of many cells and cell types with each other and the extracellular matrix. A constant exchange of information is being processed between the endothelium, the extracellular matrix, cytokines and growth factors in the extracellular milieu, and surrounding cells. These cellular events lead to the ultimate organization of endothelial cells, matrix and supporting cells into properly oriented lumen-containing structures. All these components will be discussed in detail later. Large vessels then recruit smooth muscle cells and microvessels generally recruit pericytes, mesenchyme-derived smooth muscle-like cells, which support and stabilize vessel structure. Once vessels are lined with such supporting cells the vasculature becomes a stable, mature vessel. At some point in development, capillaries also acquire tissue-specificity in addition to endothelial specificity based on expression of cell surface-associated antigens detected in mature vessels. This tissue-specificity of vascular beds was shown for brain, ovary and lung endothelium, each of which express antigens primarily expressed on cells

of their respective tissues in addition to endothelial-specific antigens (Auerbach et al., 1985). This complex structure and differentiation begins to suggest organ-specific variations in the angiogenic process that might have implications for the existence of differences between developmental and pathological angiogenesis.

What is the precise cellular mechanism of angiogenesis? Is a stepwise procedure followed? To answer these and many other questions, *in vivo* and *in vitro* assays have been developed. *In vivo* experiments most generally assay an endpoint in the angiogenic process, tubular structures assessed histologically and by in situ analysis after removal from the animal. Perfusion of dyes or injection of acrylic monomers to make vascular casts allows visualization of vessel structure over a large space (Phillips et al., 1991; Springer et al., 2000). Local or systemic interference, or an attempt to interfere, with angiogenesis is often assessed in such *in vivo* assays. However, due to the complex nature of angiogenesis, the development of assays that isolate distinct cellular processes is vital to determining the mechanism of angiogenesis and influences on each step. *In vitro* assays have the advantage of isolating and examining a particular step of angiogenesis; yet each assay, *in vitro* or *in vivo*, has its own caveats. Assays *in vivo* take advantage of the complex microenvironment of the vasculature. On the other hand, this complexity makes it extremely difficult to dissect the influence of individual molecules on the overall process. *In vitro* assays provide an environment in which individual components can be controlled, adjusted and interfered with. Conversely, this apparent simplicity potentially omits critical players in angiogenesis and can oversimplify the process leading to misleading conclusions. In order to draw accurate conclusions

regarding the mechanism of angiogenesis, both *in vitro* and *in vivo* data need to be considered.

Assays

As a first assessment of many putative angiogenesis inhibitors, investigators look at their ability to inhibit endothelial cell proliferation. In a very well defined medium, cells in culture can be treated with specific doses of effectors and proliferative index determined. Changes in the rate of endothelial cell proliferation correlate well with defects in vasculogenesis and angiogenesis (Harrington et al., 2000; Namba et al., 2000). Migration of endothelial cells is likewise a necessary step for the progression of angiogenesis and interference with this can inhibit overall angiogenesis (Ziche et al., 1994). In order to assess the migratory ability of the cell a host of assays can be performed. Some classic assays include measuring uptake of colloidal gold as cells move on this substrate (Albrecht-Buehler, 1977; Zetter, 1980), migration across a membrane or migration into an artificial wound (Fischer et al., 1990). These relatively straightforward proliferation and migration assays have prognostic value but must always be used in conjunction with *in vivo* assays to determine the real effect on angiogenesis of any agent being tested.

Endothelial cells in culture can be assayed for a more complex set of cellular events as well. In many variations of a basic tube-forming assay, the potential to form a network mimicking the vasculature can be assessed. Most such assays involve plating of endothelial cells either in or on a three-dimensional matrix and analysis of the number or complexity of linear structures. The detail and variety within this type of assay is

extensive. Plating of endothelial cells at high density can be sufficient to observe spontaneous alignment into cord-like structures. Spontaneous tube formation most likely involves cell migration as well as some intercellular signaling to arrange into lumen-containing structures. Other techniques require both this migration and organization as well as proliferation of cells. A defined number of endothelial cells can be attached to microcarrier beads and suspended in a three-dimensional matrix (Nehls and Drenckhahn, 1995a; Nehls and Drenckhahn, 1995b). From this initially attached and limited population, cells must expand in cell number, migrate and form sprouts. Conceptually similar to this is an assay for vascular channel formation or subsequent sprouting induced in embryoid bodies, an aggregation of embryonic stem cells, imbedded into a matrix gel (Feraud et al., 2001; Wang et al., 1992). The variations on tube forming assays are as numerous as the number of matrix molecules, types of cultured endothelial cell lines and environments, combined. These assays do look at the coordination of proliferation, migration and organization but importantly lack at least one major constituent, neighboring cells and the associated signaling that may take place amongst these cells.

D'Amore and colleagues have developed co-culture assays which test both simple and complex steps of tube formation. Preliminary work developed assays to test directional migration of endothelial cells and pericytes towards each other, representing the recruitment that must take place in maturing vessels (Hirschi et al., 1999). Using fluorescently labeled pericyte precursors and endothelial cells, the D'Amore lab has also developed an assay to assess proliferation of these cells in co-culture, another important process in vessel development. Much more complicated three- dimensional assays took years to develop, involving both vascular cell types to generate endothelial cell tubes

surrounded by differentiatied mesenchymal precursor cells, representing pericytes. Endothelial cells labeled with a vital red fluorescent dye and 10 T 1/2 cells (mural precursors) labeled with a vital green fluorescent dye are plated onto Matrigel®® and in 18 hours migrate and organize into vascular structures. These structures have been analyzed by EM and contain lumen as well as complex junctions.(Darland and D'Amore, 2001). These assays will be invaluable to investigating potential players in vascular remodeling, stability and regression.

The rat aortic ring model is an "*ex vivo*" model in which complex interactions contribute to the final readout. The assay measures cell outgrowth from an aortic segment, including multiple cell types, imbedded into three-dimensional matrix. (Nicosia and Ottinetti, 1990a). This model has the advantage of being an *in vitro* system which examines vessel development under chemically defined culture conditions, yet includes more than just an endothelial component. The structure and composition of sprouts from rat aortic rings in various matrices; Matrigel®, collagen, fibrin, and plasma clot, were compared and variations were found in proliferation, number of cords, number of lumenal structures and lumenal diameter (Nicosia and Ottinetti, 1990b). To take advantage of transgenic animals, I will describe in this thesis modifications of this assay and its use with mouse aortic segments.

All *in vitro* tube-forming assays have the same drawback that, in the absence of further analysis, it is impossible to determine if cord-like structures in fact contain a lumen. As Nicosia and Ottinetti found angiogenic differences in outgrowth from aortic rings in various matrices, other tube-forming assays must assess potential differences in multiple matrices. Addition of putative anti-angiogenic compounds in such assays have

shown an accurate correlation with *in vivo* data proving that despite the lack of proper vessel structure, these assays provide a generally valid method for testing for an angiogenic response.

Several assays have been developed in which defined components in angiogenesis can be introduced into a fairly normal and highly angiogenic environment to assess their influence on the process. Mammalian and avian whole embryo cultures have been used primarily to study short-term effects of agents or of transgene expression, with the focus on vasculogenesis and organ formation (Feucht et al., 1997). The *in vitro* chick chorioallantoic membrane (CAM) system extends the capabilities of *in ovo* procedures in multiple ways. The assay provides an environment in which the embryo can be maintained for extended periods *in vitro*. The CAM forms soon after explantation, leaving adequate time (6-9 days) for assessing subsequent angiogenic responses. In that time, growth can be continuously monitored and response can be photographed at multiple stages. Additionally, experimental and control grafts can be placed on the same membrane, reducing variability and providing an excellent control (Auerbach et al., 1974). Unfortunately, the expansion of CAM vasculature can itself obscure quantification of growth. De novo vessel growth, on the other hand, is more easily quantified in the normally avascular cornea. The corneal micropocket assay originally developed in the rabbit (Gimbrone et al., 1974) induces microvascular growth from the limbal vessel in response to imbedded factors at a defined distance from the limbus. The method was adapted for use in mice, in order to take advantage of transgenic animals, available reagents for this species and the vast body of reported literature to build upon. (Muthukkaruppan and Auerbach, 1979; Muthukkaruppan et al., 1982) A priori, there is

suspicion of substantial microenvironmental differences in avascular tissue and the effects of those differences on the outcome of assays is unknown. This drawback is offset by the ability to test angiogenic inhibitors or stimulators systemically, orally or topically.

Another method to measure neovascularization in animals is to exploit or create an avascular region and then induce vessel growth simply by changing oxygen levels (Smith et al., 1994). Retinal neovascularization is just such a model for the ischemic disease, retinopathy of prematurity, common in neonates needing oxygen upon birth. Postnatal mice (day 5-7) are incubated for 5-7 days in hyperoxic conditions (75%) which creates an avascular region in the eye. Switching at this time to normoxic conditions induces neovascularization in the eye which can be assessed at various time points and allows for intervention with various treatments (Friedlander et al., 1996). A fluorescein-dextran perfusion method provides complete visualization of vascular patterning in flat mounted retinas. This method tests yet another type of vascular development and may represent "normal" vascular development or may provide insight into a pathological vascularization process.

As one might expect, the desire to understand the mechanism of tumor growth and vascularization is paramount to assessing the effects of modulators of angiogenesis. One must therefore be able to effectively analyze the degree of vascularization in various tumor models and successfully develop methods to inhibit it. Tumors can be formed by the injection of tumor cells subcutaneously, intravenously or orthotopically transplanted. In addition, human tumor samples or cells can be implanted into immunologically deficient animals (xenograft) to test the effectiveness of humanized reagents. Tumor

growth, a measure of its aggessiveness, can also be indicative of its ability to initiate the angiogenic switch and properly vascularize itself. Systemic treatment with inhibitors of angiogenesis hopefully, and often do, limit the growth of tumors. Therefore, tumor size can be measured non-invasively over a period of time to assess the effects of genetic variation or treatment. It is important to evaluate tumors histologically to truly assess the deveopment of the vasculature. PECAM-1, CD31, positive structures per high powered field in tumor thin sections is often used as one method of assessing vascular growth.

The RIP TAg model of tumor growth was developed by Doug Hanahan to assess an inheritable tumor model (Hanahan, 1985). Expression of SV40 LgT Antigen in a pancreatic beta islet cell type specific fashion induces highly vascularized pancreatic tumors. Isolation of islets at various stages provide internal positive controls of angiogenesis and provide a useful opportunity to compare non angiogenic islets and angiogenic islets. This model effectively monitors the switch from hyperplasia to neoplasia through the induction of angiogenesis (Folkman et al., 1989).

An alternative way to assess angiogenesis *in vivo* is to quantify the growth of vessels into a solidified subcutaneous Matrigel® plug from the host. This measures de novo growth into a previously avascular region in response to a directed stimulus. Matrigel®, a laminin-rich matrix solution isolated from Engelbreth-Holm-Swarm (EHS) sarcoma, is a liquid at 4°C and solidifies at 37°C. This property allows reagents to be suspended and locally injected into animals where the solution solidifies very quickly. Growth for several days is sufficient to allow angiogenesis to occur if pro-angiogenic stimuli are provided in the Matrigel® suspension. Growth factors, tumor cells, as well as potential anti-angiogenic compounds can all be assayed for their effect on vessel growth

in this assay. Additionally, animals may be treated systemically with investigational reagents to determine their effects. Angiogenesis into the Matrigel® plug can be measured by histological analysis or by quantifying total hemoglobin levels. Plugs, although injected as a constant volume, do not form identical 3-dimensional structures, which complicates the readout of this assay. Sections are generally stained with hemotoxylin and eosin which stains infiltrating cells, endothelial cell as well as immune cells. A more clear identification of vessels can be achieved with a histochemical reaction specific to an endothelial antigen. Furthermore, histological assessment of plugs can be time consuming whereas hemoglobin measurements cannot distinguish blood origin or vessel distribution. Several modifications of this assay have been reported in the literature or at meetings. Many aim to minimize variation in size and maximize ease of manipulation after excision from the animal, this includes modified permeable chambers implanted subcutaneously or sponge inserts implanted surgically into previously injected Matrigel® (Wood, J. , presented AACR 2/2001). Although not widely reported in use, these modifications certainly have the potential to eliminate several caveats of this experimental system.

Current methods to study angiogenesis are much improved yet the nature of the vessel components and architecture complicate studies. Work in the complex *in vivo* system utilizes the presence of multiple cell types yet undefined microenvironmental effectors and possible inflammation are less predictable. At the other extreme, most *in vitro* systems isolate only one cell type although it is assayed in highly defined culture conditions and without unknown effectors. The few assays that attempt to compromise between these two situations have been developed and most are complicated. At present

no single assay can adequately deal with all issues involved in angiogenesis and it is important to use multiple assays to validate results.

One aim of my project was to assess the angiogenic potential of beta 3-deficient endothelial cells in the context of complimentary assays in β3 -/- animals. Developing a reproducible and reliable isolation procedure to harvest primary deficient endothelial cells was the first objective. Initially with these cells, we were testing the hypothesis that minor defects not grossly evident in animals lacking this integrin would be uncovered in one or many *in vitro* assays. There was the potential that in at least one of the assays described above, defects suggested by previous beta 3 inhibitor studies (see discussion below) could be recapitulated in the genetically deficient cells. As tumor studies in the β3 -/- animals were carried out, it became clear that the phenotype of deficient animals, even in pathological angiogenesis, does not mirror the effects seen in inhibitor studies. These results raised new questions regarding angiogenesis which could be assayed *in vitro* to determine effectors of this process in β3 -/- cells. As will become clear, the results led to new conclusions about the functions of these inhibitors and the role of beta 3 integrin in angiogenesis.

Endothelial Cells

Vascular endothelial cells function beyond angiogenesis contributing to tissue homeostasis, fibrinolysis, and coagulation; blood-tissue exchange, the regulation of vascular tone, blood cell activation and as guardians of cellular extravasation (Risau, 1998). The collection of functions endothelial cells carry out in the process of angiogenesis is one of the most important roles of endothelial cells. It is their

proliferation, adhesion, and migration, their ability to digest and reassemble the ECM, and their ability to recruit supporting pericytes on which successful vessel formation depends. Without these functions there would be no need for any subsequent activity of endothelial cells.

Endothelium lines all blood vessels, yet all endothelial cells are not identical (Auerbach et al., 1985). In the normal adult animal, endothelial cells are extremely quiescent, with only 1 in every 10,000 cells (0.01%) going through cell division at any given time (Engerman et al., 1967; Hobson and Denekamp, 1984); many fewer than most other organs aside from CNS. There is great heterogeneity, structurally as well as functionally, among various vascular beds (Gerritsen et al., 1993). The intercapillary distance (ICD) varies among organs and with blood flow and pressure, presumably through oxygen sensing to ensure proper oxygenation of all tissue (Morff, 1988). Studies in tumors suggest that the distance that oxygen diffuses from a vessel and can support cell viability was 100-200μm (Folkman et al., 2000). Again in support of the importance of a dynamic vasculature, vessels are able to reorganize themselves to respond quickly to changes in oxygen supply and tissue damage. This plasticity potentially underscores the ability of the vasculature to adapt and may be critical in understanding the effects of the loss of β3 integrin.

Endothelial cells can be divided mainly into those which line large vessels or those which form small vessels (reviewed in Gumkowski et al., 1987). Large vessel endothelium is not known to be involved in angiogenesis or blood-tissue exchange of oxygen and nutrients. The microvasculature, or small vessels, which is responsible for these functions, can be further divided based on morphology into continuous, fenestrated

and discontinuous. Definitions of the morphological differences in the microvasculature are based on the permeability of the vessel. As the name implies, continuous endothelium is the least permeable. Fenestrated and discontinuous endothelium is distinguished by pore size in the endothelium and the presence of a diaphragm (fenestra) closing the intracellular gap or not. Discontinuous endothelium can be found in sites of high levels of fluid exchange (Levick and Smaje, 1987), for example in the endocrine glands, kidney, liver, bone marrow and spleen. There are also categories of specialized endothelium that have been extensively studied, including that found in high endothelial venules and that which contributes to the blood brain barrier. High endothelial venules are found in lymphoid organs and characteristically permit high levels of lymphocyte extravasation (Girard et al., 1999) and endothelium of the blood brain barrier, through receiving many environmental signals, is incredibly impervious to transendothelial migration (Balabanov and Dore-Duffy, 1998; Janzer and Raff, 1987). Even small molecules capable of transversing most endothelium are not able to get through the blood brain barrier. Evidence supports the model that environmental cues provided to the endothelium result in the assumption of specialized characteristics. This is true for fenestrated endothelium (Roberts and Palade, 1995), the blood brain barrier (Holash et al., 1993; Janzer and Raff, 1987) and cells in culture (Ager, 1987; Milici et al., 1985; Risau, 1991b; Rubin et al., 1991). Again, these examples of endothelial cell responsiveness suggest the potential for compensation in genetically altered animals to maintain a functional vasculature.

Large vessels have been categorized into venules or arterioles distinguished initially by functional observation and later by differential expression of transmembrane

proteins. Veins characteristically have low shear rates, low oxygenation and low blood pressure whereas arteries are characterized by high shear rates, high levels of oxygenation and high blood pressure. Members of the Eph receptor tyrosine kinase family and their transmembrane ephrin ligands and perhaps other receptors are differentially expressed between these two vessel types. Ephrin-B2 ligand demarcates the arteriole subpopulation while EphB4 is reciprocally expressed on venules (Wang et al., 1998). The interaction of this pair of transmembrane molecules is thought to be critical in establishing venous/artiole boundaries. These markers are expressed during early embryonic angiogenesis on the endothelium and their expression patterns extend to include the surrounding smooth muscle in an identical distribution as is seen on the endothelium (Gale et al., 2001). Based on this marker heterogeneity, Gale (2001) also recently reported the first evidence that capillaries may also differentiate into arterioles or venules. The ephrin B-2-deficient animals show defects in early vascular development, unable to remodel veins from the primary capillary plexus, with defects in establishing properly branched structures. These defects extend to affect arteriole remodelling as well (Wang et al., 1998). Targeted disruption of EphB4 essentially phenocopies the ephrin B2 mutation confirming the importance of the bi-directional signaling for proper vascular development (Gerety et al., 1999).

In addition to the many differences seen between vascular beds in development, the mature vasculature in any given vascular bed can exist in at least two distinct states. Endothelium maintains a quiescent phenotype until a precipitating event initiates an activated state. Alterations in shear stress on the endothelium, due to changes in vascular pressure, can activate NFκB inducible cell adhesion molecule expression. Activated

resident macrophages can produce cytokines and cause release of bacterial endotoxin which can also induce cell adhesion molecules. And pathological events activate endothelium either to become more adhesive or to proliferate, migrate and initiate angiogenesis. The activation of endothelium by tumors turns the angiogenic switch to on.

The tumor vessels usually sprout from the terminal portion of the terminal host arterioles although preexisting capillaries and venules neighboring implanted tumors also dilate and become more tortuous contributing to the oxygen and nutritional needs of the tumor (Hori et al., 1987; Hori et al., 1990). In contrast, using similar methods, Peters et al provide evidence that changes in vessel number, length and diameter are more pronounced in capillaries and venules with hardly any changes in arterioles (Peters et al., 1980). More recent or definitive data on the specifics of the vascular origins of tumor cells is lacking at this time.

Regardless of what vascular beds are involved, the expansion of the endothelial population is necessary for vessel growth. Endothelial proliferation is dependent on growth factors which will be discussed below. These growth factors are produced directly by tumors cells as well as by perivascular cells and the endothelial cells themselves as a secondary event to cytokines released by the tumor.

Perivascular Cells

In nascent vessels, after the organization of endothelial cells into tubes, remodeling occurs. The integrity of the microvasculature is dependent on the presence of supporting cells, which encircle the vessel to stabilize it structurally. Pericytes, or mural cells, are defined as the microvascular equivalent of classical smooth muscle cells (SMC)

which are found surrounding larger vessels. The exact developmental cues involved in pericyte differentiation are still not clearly defined and several groups are currently working towards a better understanding the characteristics of this cell. Currently, two markers are commonly used to identify and define pericytes, NG2 proteoglycan and smooth muscle actin. Pericyte, coverage of endothelial cells is variable, dependent on the vascular bed in which they are found. Pericytes generally do not entirely cover the ablumenal surface of vessels but do extend long processes interdigitating with and wrapping around endothelium. In addition to the structural stability provided by these supporting cells, much evidence has been accumulated which implicates these cells in regulation of vascular function, including coagulation and atherosclerotic plaque formation. As mentioned above, in mature vessels, these cells also contribute to the activation of the endothelium (Reynolds and Redmer, 1998). Perivascular cells can produce a host of secreted factors which can interact with endothelial receptors and affect activity. Interestingly, though as you would expect, many factors inhibit continued angiogenesis and thus would promote vessel stabilization. TIMPs produced by SMC and pericytes (discussed below) abrogates endothelial membrane associated MT1-MMP activity (Lafleur et al., 2001). Pericytes also produce Ang-2 supporting endothelial cell differentiation and stabilization, possibly, but not definitively, antagonizing Ang1 activity (Teichert-Kuliszewska et al., 2001). Tight juxtaposition of supporting cells with the endothelium is critical for vascular integrity. Recent evidence has indicated that defects in radial glial cell adhesion to brain endothelium contributes to the lethality of αv deficiency in mice (McCarty, J. submitted).

Macromolecular Components of Vasculature

As I implied in the previous section, the functions of the vascular cells are dependent on the functions of the proteins expressed in those cells. The scope of this thesis will limit my discussion to adhesion and growth factor-related molecules effecting the morphogenesis of blood vessels. Structural elements such as the extracellular matrix and the molecules that engage matrix (integrins) and degrade matrix (proteases) will be included. I will introduce to you, the reader, the major players involved in proliferation, migration, survival and death of the endothelial cells contributing to the adult vessels.

Integrins

Integrins are a family of heterodimeric transmembrane cell adhesion molecules which can mediate the interaction of cells with extracellular matrix and neighboring cells. Extracellular matrix (ECM) is a network of proteins which act as the scaffold of the extracellular space giving structure to differentiated tissues and organs. ECM is dynamic and its composition changes during tissue formation accompanied by alterations in cell growth and migration. Integrin/ECM interaction is known to stimulate a multiplicity of functions in cells. Basic cellular activities such as attachment, spreading, migration, proliferation and death can all be influenced by integrin/ECM interactions. These processes are critical for normal as well as pathological processes including embryonic development, morphogenesis, tissue remodeling, wound healing, thrombosis, inflammation, tumor progression and metastasis.

The integrin family functions when a partnership is formed between 1 of 18 functionally identified alpha subunits and one of 8 functionally identified beta subunits

and this molecule is expressed on the cell surface. 24 functional integrin heterodimers have been identified and can be divided into subgroups based on their subunit composition and ligand recognition (see Table 1.1). Distribution and structure of alpha and beta subunits is conserved in human and mouse. Each heterodimer has a characteristic ligand-specificity albeit often including several ECM ligands and each ECM protein has multiple integrin receptors.

Common features exist in several alpha and beta subunits. Alpha integrins vary from ≈140kD to 210kD and beta integrins (excluding beta 4 which is substantially larger due to an unusually large cytoplasmic domain) are ≈90-130kD. All alpha subunits contain a conserved GFFKR (or very similar) protein sequence in their cytoplasmic tails proximal to the transmembrane region. Many beta subunit cytoplasmic tails have at least one NPXY sequence which may be important for integrin localization, endocytosis and signaling (Hsu et al., 1994). Beta subunits also have conserved cysteine-rich repeats in their carboxy terminal half. Recently the first crystallographic data on a whole integrin molecule has been reported for the αvβ3 integrin heterodimer (Xiong et al., 2001). As predicted by Springer (1997), the alpha subunit contains an amino-terminal 7 unit β-propeller motif similar to G-protein β domains. The alpha v subunit lacks an I domain, a 200 amino acid insertion, common to many alpha subunits but does have a disulphide-linked cleavage site in the juxtamembrane portion of the extracellular domain (Lin et al., 1997). The I domain, or VWA domain, can be found in α1, α2, α10, α11, αm, αx, αd, αl, αe and can function independently binding divalent cations and ligand through a MIDAS (metal ion dependent adhesion site) motif (α2β1 in Dickeson et al., 1998;

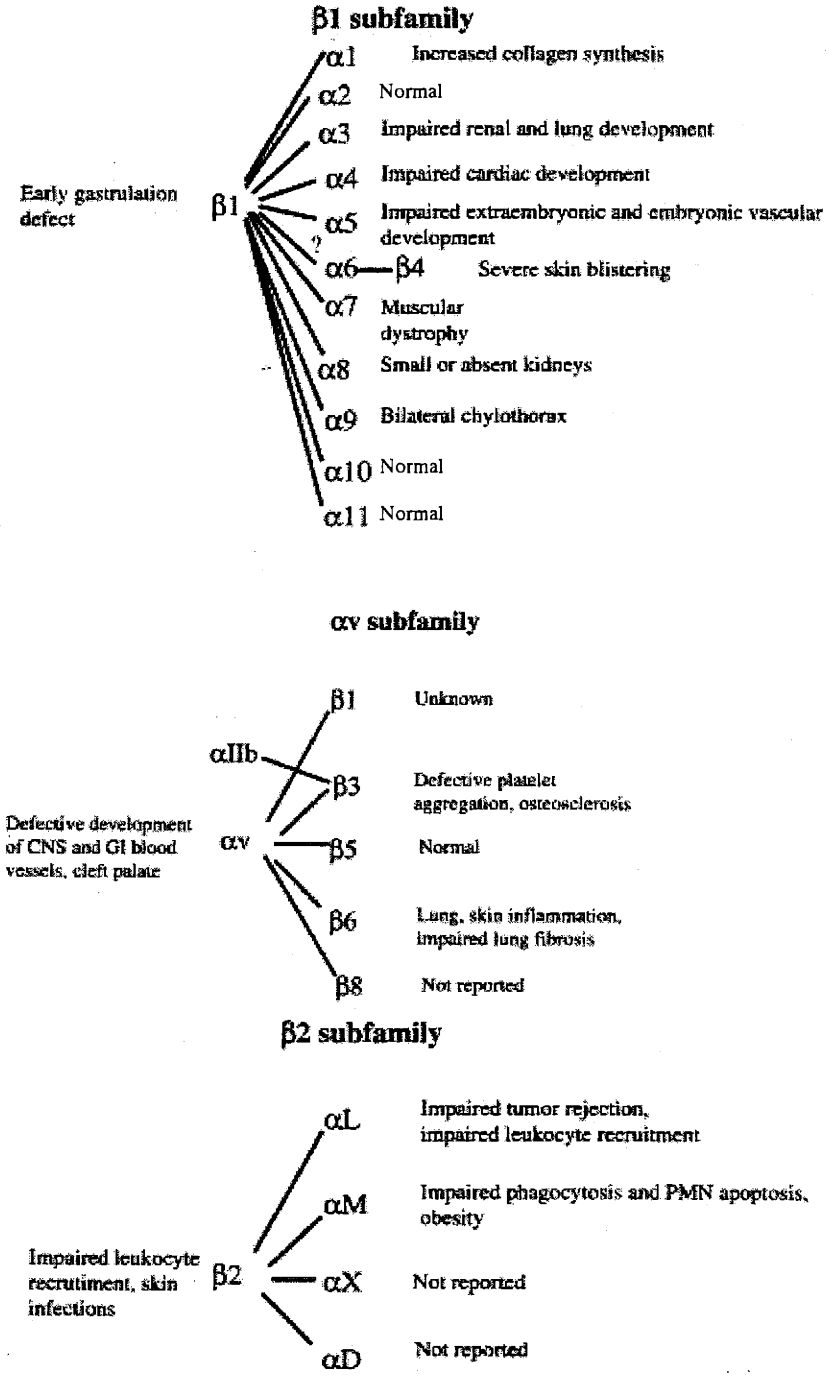
Gotwals et al., 1999; and Lee et al., 1995a; αmβ2 in Lee et al., 1995b; α1β1 in Nolte et al., 1999) (reviewed in Kanazashi et al., 1997; Plow et al., 2000)

Categorization of integrins is based on their affinity for certain ligands or as for beta 2 integrins, their tissue distribution. The subgroup consisting of αv integrins, αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8 primarily recognize the RGD (Arg Gly Asp) motif found in several extracellular matrix molecules including fibrinogen, vitronectin, fibronectin, osteopontin, tenascin as well as the non-RGD ligand von Willebrand Factor. The targeted disruption αv integrin has an interesting yet complicated phenotype. The majority of mice die embryonically of hemorrhage in the brain (Bader, 1998). The disruption of αv partners β3, β5 or β6 individually do not lead to a similar phenotype and all are viable and fertile (Hodivala-Dilke et al., 1999; Huang et al., 2000; Huang et al., 1996). In fact, animals lacking both β3 and β5 or β3 and β6 likewise are viable and fertile (pers. comm. R.O.Hynes). However, the phenotype of animals lacking β8 integrin is very similar to the αv-null phenotype suggesting the most critical αv integrin in development may be αvβ8 expressed on non-endothelial cells (pers. comm.). More details will follow at the end of this introduction on αvβ3 integrin, the focus of this thesis. In addition, several other integrin heterodimers recognize the RGD sequence including α5β1 and α8β1. α1β1 and α2β1, both found on endothelial cells, are major receptors for various collagens, as well as α10β1 and α11β1. The subgroup of α3β1, α6β1, α7β1, and α6β4 integrins comprises the primary laminin receptors. β2 integrin subunit partners with 4 alphas, αm, αl, αx, and αd and these heterodimers are expressed exclusively on leukocytes. As a family they mediate cell-cell adhesion and bind primarily to IgG superfamily members, ICAM-1, -2, or -3, iC3b (complement) and

fibrinogen. These integrins participate in leukocyte homing, recruitment and recirculation.

Table 1: Integrin Family Membersand Knockout Phenotypes

These trees show alpha and beta heterodimers which can form and also list the phenotype of genetic ablation of each subunit that has been reported (from Sheppard, 2000).



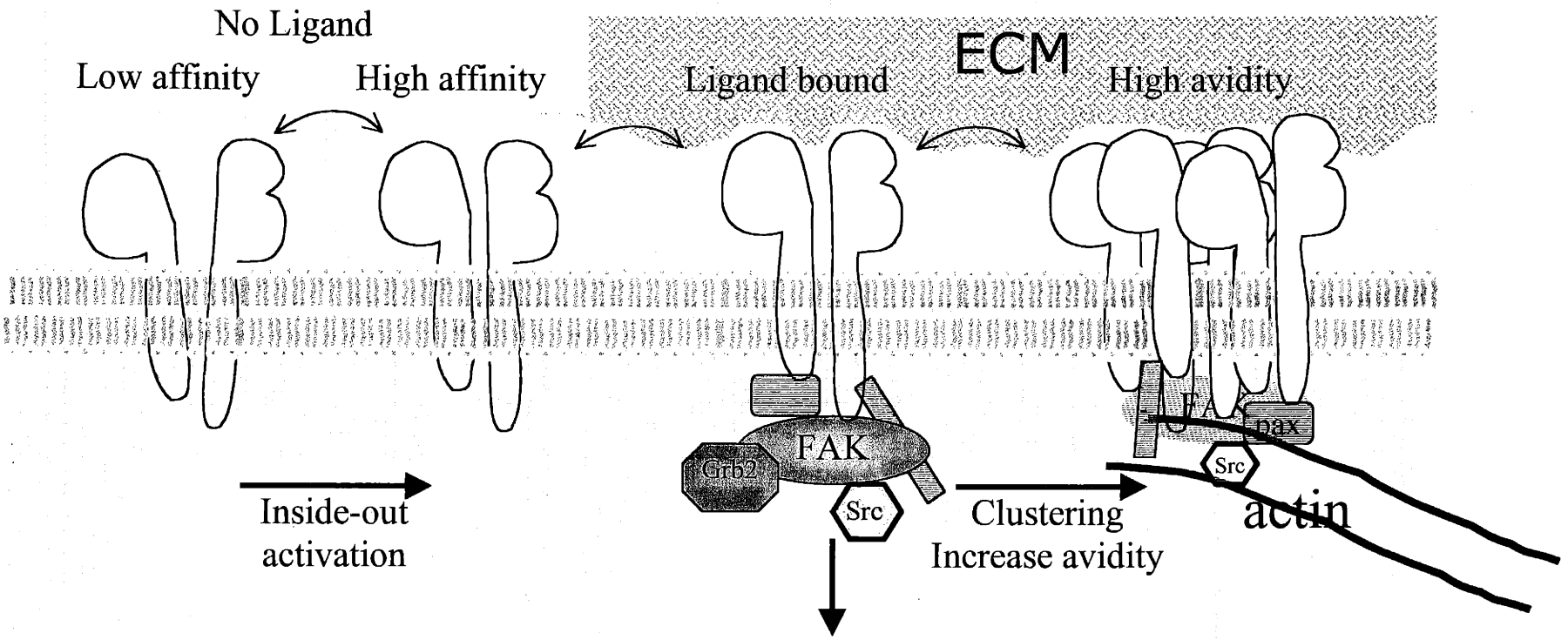
One of many functions of integrins is to serve as a linking molecule between the outside and the inside of the cell. In addition to binding a host of extracellular molecules, the integrins ability to bind molecules through their cytoplasmic domains is essential for integrin function. Intracellularly integrins interact with the scaffolding of the cell, the cytoskeleton; extracellularly they interact with the tissue scaffolding, the extracellular matrix. These interactions result in signals which are transmitted by the integrin in both directions, outside-in and inside out. Signals from the extracellular environment (outside-in) lead to cellular decisions such as proliferation, migration and apoptosis. Inside-out signaling creates a conformational change in the integrin and to affect its affinity for and ability to bind ligand (Ginsberg et al., 1992).

There are several conformations which affect integrins' ability to bind ligand and transmit signals (Fig. 1.2). Unliganded integrins are generally distributed over the entire cell surface and appear not to be linked to the actin cytoskeleton (CSK) through their cytoplasmic domains. Association with the CSK is induced upon binding to ECM ligands. Integrins can then be clustered into specialized adhesive structures, focal adhesions (FAs) and focal complexes, in which numerous signaling components are concentrated (Schoenwaelder and Burridge, 1999). In particular, the integrin beta-subunit can be linked to F-actin via the actin-binding proteins talin, alpha-actinin and filamin (Critchley et al., 1999). Large integrin clusters can be found at the tips of stress fibers (bundles of actin filaments) in FAs or at edges of filopodia and lamellapodia (cell membrane extensions) in focal complexes. Both structures are regulated by distinct members of the Rho family of small guanosine triphosphatases (Hall, 1998). Focal complexes can develop into focal adhesions through the recruitment of additional

associated intracellular molecules or otherwise dynamically disassemble. Very large signaling complexes are generated in focal adhesions including many SH2 containing proteins, Src family kinases and PI3K, as well as tyrosine phosphorylated proteins, focal adhesion kinase (FAK) and paxillin, to name a few (Guan, 1997). These signaling components are recruited, localized and activated at the membrane proximal region upon integrin ligation and are responsible for regulating cell survival, proliferation, migration and apoptosis.

Figure 1.2: Schematic of Integrin Activation States and Intracellular Partners

Integrins exist in multiple states (probably different conformations) which are affected by both inside-out and outside-in signaling. From a low-affinity, unbound state, inside-out signaling events create a high-affinity but unbound form. Binding of ligand induces outside-in signaling and the recruitment of intracellular signaling partners. Association of actin cytoskeleton induces clustering and high avidity ligand binding.



Matrix

Matrix is a dynamic component of the vasculature. As intimated above, the interaction of integrins and matrix is complex due to the sheer number and variety of ligand/receptor interactions that take place. Quiescent vessels are surrounded by and supported by a vascular basement membrane, a specialized highly organized extracellular matrix, primarily but not exclusively, composed of type IV collagen, discussed in a devoted section below, laminin, heparan sulfate proteoglycan perlecan, and stabilized by nidogen cross-linking (Paulsson, 1992). "Active" vasculature, initiated by injury or in pathology, is supported by a provisional matrix composed mainly of fibrin, fibronectin, vitronectin and von Willebrand Factor (Clark et al., 1996).

Collagens are a large family of triple helical molecules. Each of three subunits (alpha chains) contains a $(Gly\ X\text{-}Y)_n$ repetitive sequence motif which provides flexibility in the collagenous region. Collagens are divided into several subfamilies including fibrillar collagens, basement membrane collagens, short chain collagens, FACITs, MACITs and multiplexins (reviewed in Olsen, 1999). Domain structures vary amongst these subgroups but can include non-collagenous domains (NC) and globular domains. Collagens characteristically form mulitmeric networks, fibrils or filaments in complex with themselves or other extracellular matrix molecules.

Different collagens can be cleaved by different matrix metalloproteinases. Collagen IV is cleaved to yield three-quarter and one-quarter length fragments freeing the NC1 domain from the helical region (Murphy and Crabbe, 1995). NC11 of Collagen XVIII, is preseumably likewise cleaved from ths triple helix and may play an interesting

role in the endogenous vasculature as an inhibitor of angiogenesis. Recombinant production of this domain has proven to be an active antiangiogenic molecule. NC1 domains of several different Type IV Collagen alpha chains are proving to be potent inhibitors of angiogenesis. Collagens will be discussed in detail below in conjunction with a broad spectrum of angiogenesis inhibitors.

The heterotrimeric family of laminins is another major constituent of the basement membrane found in quiescent vessels. An alpha, beta and gamma subunit combine to form 1 of 11 identified laminins. Laminin-1 was the first component of EHS tumor basment membrane (ie Matrigel®) purified. Laminins complexed very tightly with nidogen contribute to collagen IV binding in basement membranes (Paulsson et al., 1987). Laminins also interact directly with Collagen IV, other extracellular ligands and cells through both integrin and non-integrin receptors. Isoforms are broadly expressed with overlapping, but restricted, distribution.

The provisional matrix molecules provide essential regulatory signals for endothelial cell adhesion, proliferation, and gene expression. A major constituent of provisional matrix is the molecule near and dear to the heart of our lab, fibronectin. The structure of fibronectin is a series of repeating units (FN Type I, II and III repeats) which form structural and functional domains specializing in binding to integrins and other ECM molecules. Transgenic animals lacking fibronectin are embryonic lethal at day 7.5-8 of embryonic development (Georges-Labouesse et al., 1996). The extent of their developmental defect is dependent on the genetic background in which the mutation is found. In all strains tested, animals exhibit a characteristic yolk sac defect. FN can be differentially spliced to create several variants. Specialized functions of splice variants

have not been assigned although their distribution is somewhat regulated. FN has two cell binding domains that facilitate integrin adhesion. In addition to the RGD sequence discussed above, the secondary ILDV sequence is recognized by $\alpha 4\beta 1$ (VLA-4) integrin (Huo et al., 2000).

Vitronectin, another component of the provisional matrix, contains an RGD integrin binding domain. Cells expressing any one of at least six known vitronectin receptors: integrins alpha v beta 1, alpha v beta 3, alpha v beta 5, alpha IIb beta 3, alpha v beta 8 or alpha 8 beta 1 recognize vitronectin as an adhesive substrate (Felding-Habermann and Cheresh, 1993; Nishimura et al., 1994; Schnapp et al., 1995). The vitronectin knockout had no gross developmental defects despite deficient PAI-1 binding of VN deficient serum (Zheng et al., 1995). Vitronectin is critical in fibrinolytic and coagulation pathways, binding heparin like glycosaminoglycans. An interesting report has shown that ligation of vitronectin by alpha v integrins *in vitro* can protect the microvascular EC from programmed cell death (Isik et al., 1998).

Thrombospondins constitute another family of extracellular matrix glycoproteins. TSP-1 and TSP-2 are structurally similar and represent a subgroup of thrombospondins which are trimeric and contain type I repeats (reviewed in Lawler, 2000). Each subunit of the TSP-1 and TSP-2 homotrimers contains conserved structural and functional domains. Type 1, type 2 and type 3 repeats as well as amino and carboxy terminal globular domains and a pro-collagen-like domain are common to both TSP-1 and TSP-2 and combine to create the modular structure of this subgroup of thrombospondins.

Several of these structural domains are known to contribute specifically to TSP-1 associated activity. The multiple sites of TSP-1 with diverse interactions permits TSP-1

to facilitate the colocalization of many molecules. Type 1 repeats in both TSP-1 and TSP-2 are critical for the interaction with TGF-β however only TSP-1 can activate the latent molecule (reviewed in Chen et al., 2000). The last type 3 repeat of TSP-1 interacts with αvβ3 integrin.

TSP-1 is found in the blood vessels and has the ability to interact with growth factors, other matrix molecules and matrix metalloproteinases. TSP-1 knockout animals are viable and exhibit only mild developmental defects (Lawler et al., 1998). Adult animals do exhibit extensive inflammation in the lung possibly due to the lowered TGF-β activity seen in null animals. Again similar to other molecules playing important roles in the vasculature, apparently duplicitous roles for TSP-1 have been implicated, primarily, an inhibitor of angiogenesis (reviewed in Lawler, 2000) and also an indirect pro-angiogenic activity by stimulating myofibroblast growth (Nicosia and Tuszynski, 1994). Two sequences have been shown to have the antiangiogenic activity; one in the pro-collagen homology region and another in the type 1 repeat (TSR). Assays done in the CAM angiogenesis model have not supported the claim that the pro-collagen domain contains antiangiogenic activity (Iruela-Arispe et al., 1999). TSP-1 can inhibit the activity of another important molecule in the angiogenic process, MMP-9.

TSP-2 similarly to TSP-1 is expressed at sites of tissue injury and in association with tumor growth. TSP-2 deficient mice are viable but exhibit abnormalities in collagen matrix assembly and connective tissue. The null animals also have increases vessel density in the dermis and subdermal tissue. The interactions that TSP-2 take part in are much less defined than those of TSP-1.

Matrix Metalloproteinases

The dynamic nature of matrix is at least in part regulated by a family of extracellular proteases called matrix metalloproteinases (MMPs) that are essential for proper ECM remodeling (reviewed in Vu and Werb, 2000). MMPs as a family have broad specificity for substrate. There are now more than 20 members of the MMP family. MMPs have been implicated in tumor growth, invasion and metastasis; their upregulation can be diagnostic of a poor prognosis. Several studies have demonstrated that the gelatinases, MMP-2 and MMP-9, and the membrane bound MMP, MT1-MMP, are most involved in the vasculature (Haas and Madri, 1999; Nguyen et al., 2001). Our understanding of the effects of these MMPs on the vasculature is evolving and becoming quite complex.

MMP activity is potentially regulated at three steps: (1) gene expression, (2) pro-MMP cleavage and activation, and (3) inhibition of enzymatic activity by endogenous TIMPs (tissue inhibitors of metalloproteinases) (Hidalgo and Eckhardt, 2001). MMP-2 (gelatinase A) is constitutively secreted while MMP-9 (gelatinase B) is inducible secreted by the microvasculature. Both are synthesized as inactive proenzymes which are activated by proteolytic cleavage. MMP-2 is uniquely activated by MT1-MMP and requires binding of TIMP-2 (Tissue inhibitor of metalloproteinase) for localization to the cell surface and activation. MMP-9, on the other hand, can be activated by MMP-3. MMP activity also can both affect and be affected by signaling through integrins as well as the growth factor: growth factor receptors.

Mice homozygous for a null mutation in the MMP-9/gelatinase B gene show developmental defects including abnormal vascularization of the skeletal growth plate.

In vitro assays confirmed this phenotype results from the delayed release of an angiogenic activator from deficient growth plates suggesting this role for MMP-9 in angiogenesis (Vu et al., 1998). Studies in the RIP-TAg model of tumor progression show that both MMP-9 and MMP-2 are upregulated yet only MMP-9 causes switching to angiogenic islets from normal islets. Antagonists of MMPs inhibit the angiogenic switch but only MMP-9 deficient animals in this model have reduced angiogenesis (Bergers et al., 2000). MMP-2 deficient animals however did show reductions in both tumor induced angiogenesis and tumor growth in another model (Itoh et al., 1998). Importantly this indicates the MMP involved in the progression of tumors are at least in part host derived.

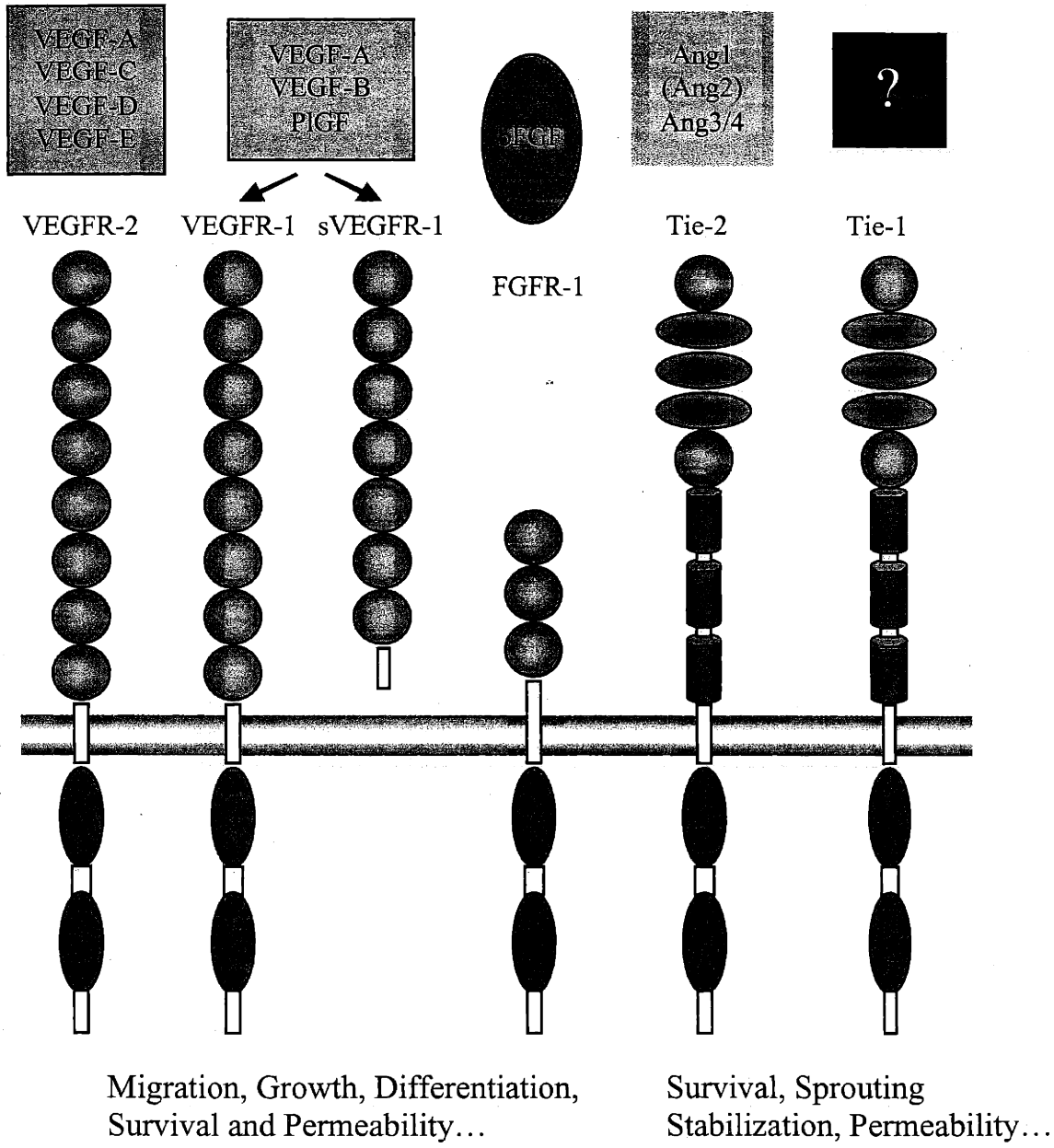
In addition to degrading the basement membrane and inducing cell invasion, MMPs also promote angiogenesis by releasing VEGF165 bound to the ECM thus stimulating proliferation and possibly migration of endothelial cells.

Growth Factors in Angiogenesis

Mitogenic control and regulation of endothelial proliferation are critical to the overall success of the angiogenic process. There is both redundancy and competition to ensure a regulated but robust angiogenic response. Aside from such ubiquitous growth factors with diverse targets, such as basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) and epidermal growth factor (EGF) (reviewed in Dunn et al., 2000)), the VEGF/VEGF-R family and Angiopoeitin/Tie ligand/receptor families play major specific roles in the regulation of angiogenesis. Regulation comes at the level of transcription, bioavailability and competition for receptor/ligand interaction.

Figure 1.3: Schematic of Angiogenic Growth Factors Structures

Growth factor receptors important in angiogenesis are depicted here. Extracellular domains are modular comprised of Ig- like (blue circle), EGF-like(purple oval) and FN Type III (green column) repeats. Intracellular domains contain kinase domains and a kinase insert (yellow box). Ligand specificity is listed above the receptor. (modified from Jones et al., 2001)



Vascular endothelial growth factor (VEGF, also known as vascular permeability factor, VPF) was initially identified in the late 1970s on the basis of its ability to induce

transient vascular leakage. Tumor ascites from guinea pigs, hamsters, and mice, as well as cell lines isolated from these tumors, were found to possess a secreted activity which rapidly increased microvascular permeability (Senger et al., 1983). The mechanism by which VEGF/VPF induced permeability however was not clear. Recent studies show that binding of VEGF/VPF to endothelial cells induces MAP kinase-dependent disorganization of intercellular junctions (Kevil et al., 1998) and phosphorylation of VE-cadherin (Esser et al., 1998). Additional studies suggest that VEGF/VPF-induced nitric oxide (NO) and prostacyclin production are responsible for the increase in vascular permeability (Murohara et al., 1998) VEGF/VPF gained its identity as a growth factor when VPF was found to promote proliferation of cultured endothelium as well as induce blood vessel growth in the rat cornea (Connolly et al., 1989).

VEGF was characterized as a heparin-binding, dimeric, disulfide-bonded glycoprotein. VEGF molecules are found primarily as homodimeric complexes although heterodimers of VEGF-A and PlGF have been isolated from a rat glioma cell line and both homo-and hetero dimers have nearly equal activity (DiSalvo et al., 1995). VEGF expression is transcriptionally regulated by hypoxia which occurs during tumor expansion and ischemia (Shweiki et al., 1992) (also post-transcriptionally through mRNA stablization (Hata et al., 1999)). In addition, the expression of VEGF is affected by activated oncogenes and inactivated tumor suppressor genes and other growth factors (reviewed in Martiny-Baron and Marme, 1995; Rak et al., 2000).

Over a dozen years of research have identified several members of the VEGF family: VEGF-A (the founding member of this family), placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and the parapox virus VEGF, referred to as VEGF-E.

Recently researchers identified a tissue-specific endocrine-gland VEGF or EG-VEGF which functionally resembles VEGF yet lacks structural homology (LeCouter et al., 2001). The importance of VEGF in vascular development is highlighted by the finding that heterozygous or homozygous VEGF-A-deficient mice suffer early embryonic lethality due to abnormalities in the vasculature (Carmeliet et al., 1996; Ferrara et al., 1996). Likewise, ablation of the receptors for this growth factor, VEGF-R 1 or 2 (Flk-1, Flt-1), causes early embryonic lethality due to vascular defects (Fong et al., 1995; Shalaby et al., 1995).

Alternative splicing of the gene encoding VEGF-A generates six variants that differ in total amino acid number. In humans, these correspond to VEGF-A 121, VEGF-A 145, VEGF-A 165, VEGF-A 183 (Jingjing et al., 1999), VEGF-A 189 and VEGF-A 206 (Cross and Claesson-Welsh, 2001) (in rodents each isoform is a single amino acid shorter). VEGF-A 164/165 is the predominant form and can be found both retained in the ECM or fully secreted. VEGF 120/121 is strictly soluble whereas VEGF 189 is only found bound to ECM. VEGF 144/145 and 205/206 are rare variants. Previously, VEGF 144/145 had been detected only in placental and uterine tissues and endometrial carcinoma cell lines, whereas VEGF 205/206 had been detected only in fetal liver and placenta. Burchardt et al (1999) were able to confirm the presence of mRNA encoding VEGF 144/145 and 205/206 in both adult rat lung and penis using an RT-PCR technique suggesting a more diverse expression pattern than was originally proposed for these low-abundance VEGF splice variants.

As tumors expand, a reduction in oxygen tension creates a new demand for the development of an efficient vascular supply. This deficiency in proper oxygenation

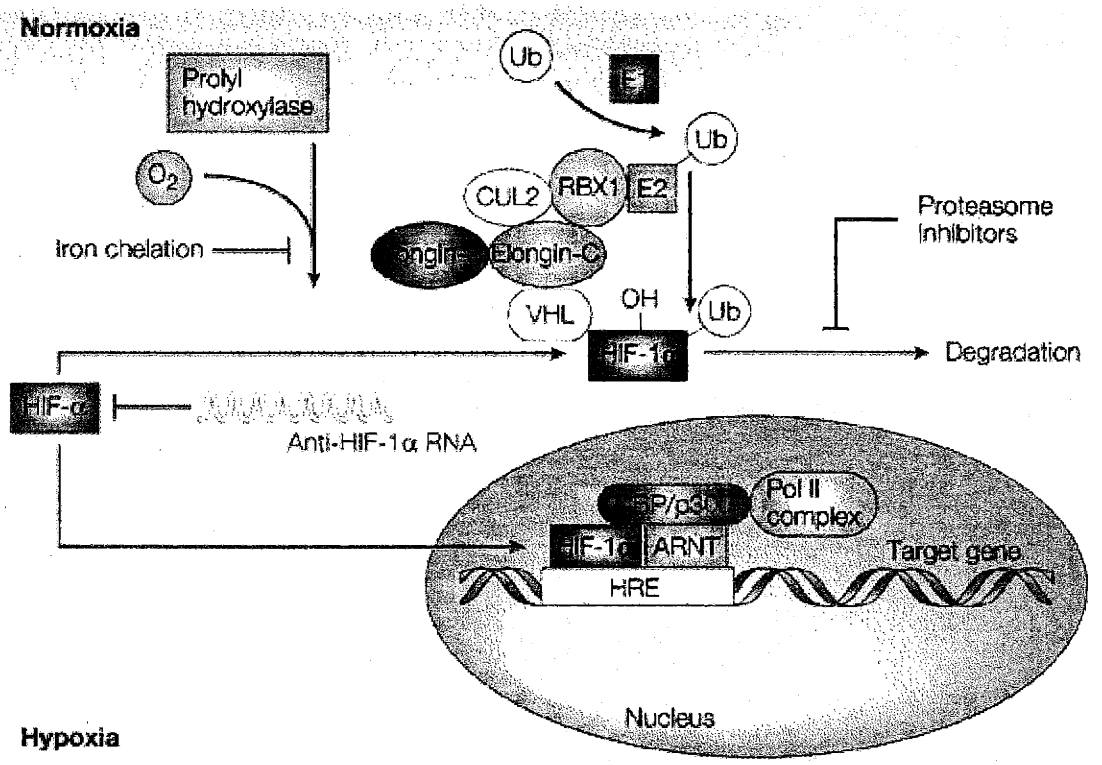
mediates signals that induce neovascularization in order to satisfy the needs of the tumor. A low oxygen level, hypoxia, initiates a string of coordinated events cascading from the activation of HIF-1, which in turn induces anaerobic metabolism (Semenza et al., 1996), angiogenesis, vasodilation, erythropoiesis (Wenger and Gassmann, 1997) and increased breathing. This involves transcription of many genes whose promoters contain a hypoxia response element (HRE) to which HIF-1 binds. VEGF is one of the many genes regulated by the HIF-1 transcription factor (Figure 1.4). VEGF messenger RNA levels are dramatically increased within a few hours of exposing different cell cultures to hypoxia and return to background when normal oxygen supply is resumed (Shweiki et al., 1992).

Tumors created from HIF-1-null cells are smaller despite normal vessel density; thus HIF-1 must have additional functions, independent of VEGF upregulation, which affect tumor growth (Ryan et al., 2000).

HIF-1 is a heterodimer of alpha and beta subunits belonging to the basic helix-loop-helix (bHLH) PAS (Per-ARNT-Sim) family of transcription factors (reviewed in Blancher and Harris, 1998; Wang et al., 1995; Wang and Semenza, 1995). The beta subunit is identical to ARNT (aryl hydrocarbon receptor nuclear translocator). The alpha subunit contains 2 transactivation domains necessary for HIF-1 activity. HIF-1 β protein is maintained at constant levels. HIF-1 α protein, on the other hand, is very unstable. In normoxic conditions, any HIF-1 α protein synthesized is degraded within 5 minutes in cells containing an active von-Hippel Lindau (VHL) gene; whereas in hypoxic conditions, HIF-1α protein accumulates.

Figure 1.4: Schematic of HIF-1 Regulated Pathway.

Under normal oxygen levels, HIF-1 α is targeted for degradation via the ubiquitination pathway. VHL acts as the F-box regulator of HIF-1 degradation. In hypoxic conditions, HIF-1 α cannot be modified by oxygen sensing machinery (prolyl hydroxylase) and remains stable, translocates to the nucleus where is interacts with HIF-1 β (ARNT) and activates transcription of genes containing a hypoxia response element (HRE). (from Harris, 2002)



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Oxygen regulation of gene transcription by HIF-1 is dependent on the presence of VHL. HIF-1 α contains an oxygen degradation domain (ODD) which is responsible for hypoxia responsiveness (Huang et al., 1998). The absence of this domain confers stability on HIF-1 α protein in normoxia and along with this stability the retention of all functions of HIF-1 α (HIF-1 β dimerization, DNA binding, and transactivation

independent of hypoxic signals). HIF-1 α also contains two PEST motifs (proline, glutamine, serine, threonine) which are signature motifs for rapid degradation. This degradation is mediated by the multiprotein ubiquitin ligase / proteasome-targeting complex containing VHL, a tumor suppressor gene product (Fig. 1.4).

In addition to VEGF, basic fibroblast growth factor, bFGF or FGF-2, is a potent inducer of angiogenesis (reviewed in Cross and Claesson-Welsh, 2001). It is one of at least 20 factors belonging to the FGF family. When released from the cells, FGF binds heparan sulfate proteoglycans (HSPGs) found within the extracellular matrix as well as on the surface of many cell types including endothelial cells and pericytes (Horiguchi et al., 1989). The ability of bFGF to bind ECM in effect creates a localized pool of growth factor which can be released upon cleavage of the ECM, an early step in angiogenesis. FGFs bind to receptor tyrosine kinases FGFR-1,-2, -3, or –4 which exhibit broad expression patterns. Binding of bFGF to FGFR-1, the primary interaction, is stabilized by the presence of heparin or HSPGs (Burgess and Maciag, 1989). Recent crystallographic data show a ligand:receptor complex is formed by 2 bFGF molecules and 2 FGF receptor molecules and identifies how an active complex is induced by heparin binding (Plotnikov et al., 1999). Homodimerization, upon ligand binding, results in trans-autophosphorylation of the FGFR and initiation of a signaling cascade. Targeted disruption of FGFR-1 (or R2) results in early embryonic lethality (Deng et al., 1994). To address the importance in vascular development, adenovirus-mediated expression of dominant-negative FGFR-1 beginning at embryonic Day 9.0 also showed lethality (Lee et al., 2000). In contrast, bFGF inactivation leads to viable animals with only minor vascular defects suggesting a redundant role for members of the FGF family (Dono et al.,

1998). It is equally possible in the bFGF knockout that there is compensation by other vascular growth factors and receptors which allow for the general survival in the absence of bFGF.

A second family of largely endothelial specific growth factors has more recently been associated with vascular development. The angiopoietins identified to date, Ang 1-4, bind to Tie family of receptor tyrosine kinases with preference for Tie-2 (TEK) over Tie-1. Ang-1 binding to Tie-2 induces sprouting, survival and stabilization of vessels and limiting permeability. Ang-2 is a naturally occurring antagonist and will modulate Ang1 activity by binding Tie-2. There is recent evidence however that Ang2 can initiate tube formation in EC but through what mechanism it is unknown (Teichert-Kuliszewska et al., 2001).

Severe defects in these receptor ligand partners underscore the important roles they play. No known specific ligand for Tie-1 has been identified, however the lethality of mice carrying mutations in Tie-1 indicate overlapping but non-redundant function in vascular development (Sato et al., 1995). Tie-2 null embryos die between days E9.5 and E12.5 from a failure to expand and maintain the primary capillary plexus (Sato et al., 1995). Ang-1 deficient animals are phenotypically similar to Tie-2 null animals and fail to develop normal vessels thus further supporting Ang-1 as the major physiological ligand for Tie-2 (Suri et al., 1996).

There is evidence that Tie-1 receptor heterodimerizes with Tie-2 receptor to modulate its tyrosine kinase activity (Marron et al., 2000a). They have also shown that proteolytic cleavage of the Tie-1 extracellular domain induces association of the cytoplasmic domain with tyrosine phsophatase and SHP2 (Marron et al., 2000b).

Inhibitor/Activators Review

Naturally occurring molecules, or fragments thereof and antibodies are being exploited for their ability to control angiogenesis in the hopes of discovering a highly specific cancer therapy with a small incidence of non-specific activity. Several therapies of this type appear to be immune to the development of drug resistance by the tumor cells, a potentially major improvement over current chemotherapies. There are several ways to think about the reagents being explored, by function, method of identification and targets. Considering the process of angiogenesis, as discussed above, there are several vulnerable aspects which, if properly inhibited, could undermine the entire tumor angiogenic process often without affecting other vascular beds starving the tumor and prohibiting growth. Experiments focus on drugs that are specifically targeted to proliferating endothelium and, once localized, exert a robust antiproliferative or apoptotic signal.

Table 1.2 Inhibitors of Angiogenesis

Various molecules or fragments of endogenous molecules which have been isolated and shown to have anti-angiogenic properties outlined.

Inhibitor	**Molecular and physiologic properties**
Proteolytic fragments	
Angiostatin	38-kD internal fragment of plasminogen (kringles 1–4); kringles 1–3 and kringle 5 also active
Arrestin	26-kD C-terminal NC domain of α 1 chain of Type IV collagen; inhibits EC proliferation.
Antithrombin (cleaved)	53–55-kD cleaved conformation inhibits endothelial cell proliferation and tumor growth in mice.
Canstatin	24-kD C-terminal NC domain of α 2 chain of Type IV collagen; Inhibits EC proliferation and apoptosis.
Endostatin	20-kD fragment of C-terminal NC domain of Type XVIII collagen; mechanism of action unknown.
Fibronectin fragments	29-kD N-terminal and 40-kD C-terminal heparin- binding fragments inhibit EC proliferation.

Prot. Frag. Cont. PEX	C-terminal hemopexin-like domain of MMP-2 inhibits EC proliferation and tumor growth in mice
Prothrombin kringle-2	22-kD prothrombin frag. isolated from LPS-treated serum.
Restin	22-kD fragment of C-terminal noncollagenous domain of Type XV collagen; 60% homology to murine endostatin.
Tumstatin	20-kD C-terminal NC domain of α3 chain of Type IV collagen; inhibits EC proliferation
Other	
Angiopoietin-2	Inhibits angiopoietin-1-mediated activation of EC tyrosine kinase receptor, Tie2; role in vascular remodeling
Soluble FGF receptor	60 to 85-kD circulating binding proteins that may regulate proangiogenic activity of FGF.
Transforming growth factor β1	25-kD inhibitor of EC growth and proteolytic activity.
TSP-1, TSP-2	450-kD platelet- and fibroblast-derived trimeric glycoproteins.
Interferons	
IFN- α	8 to 20-kD glycoproteins secreted by lymphocytes and phagocytes; inhibits EC proliferation and migration.
IFN- β	23-kD glycoprotein derived from fibroblasts and epithelial cells.
IFN- γ	20 to 25-kD glycoprotein secreted by T-cells and NK cells; cytotoxic to proliferating ECs
TIMPs	
TIMP-1	Soluble 8.5-kD collagenase inhibitor.
TIMP-2	Soluble 21-kD collagenase inhibitor.
TIMP-3	Extracellular matrix-associated collagenase inhibitor.
Interleukins	
IL-1	17-kD b-isoform inhibits FGF-stimulated angiogenesis by an autocrine pathway.
IL-4	13-kD lymphokine; inhibits basic FGF-induced angiogenesis.
IL-10	Inhibits tumor vascularity and growth, possibly by decreasing macrophage-derived angiogenic factors.
IL-12	75-kD glycoprotein; inhibits *in vivo* angiogenesis via IFN- g- and IP-10-related mechanism.
IL-18	IFN- γ-inducing cytokine; inhibits FGF-stimulated EC proliferation and *in vivo* angiogenesis.

A broad category of inhibitory molecules are bioactive proteolytic fragments of proteins which exist in the vascular milieu. These fragments represent primarily cleavage

products of extracellular matrix molecules and matrix metalloproteinases. Domains which contain antiangiogenic activity are diverse (Table 1.2). When isolated, enriched and administered in animal models of angiogenesis, these fragments inhibit proliferation and some can induce cellular apoptosis. This raises several questions; first, what is their incidence endogenously and second, assuming they are naturally occurring, what is their endogenous role? Despite any endogenous role they play, purification and administration of these fragments are proving to be promising anticancer therapies in mouse tumor models.

TSP-1, as mentioned above is found in the vasculature and was the first identified endogenous inhibitor of angiogenesis (reviewed in Lawler, 2000). Despite subsequent evidence of a pro-angiogenic activity (Nicosia and Tuszynski, 1994), reports clearly show that TSP-1 can inhibit endothelial cell proliferation and migration. Studies isolated the anti-angiogenic activity to the type 1 repeats of TSP-1 (TSR) and this activity was sustained in synthetic or proteolytically derived TSP-1 fragments. The amino terminus as well as the carboxy terminus of TSR2 and TSR3 both independently inhibit bFGF driven angiogenesis in the CAM model, whereas the carboxy terminus only was found to inhibit VEGF-induced neovascularization. In the rat corneal pocket angiogenesis assay and chick CAM angiogenesis assay, TSP-1 fragments were found to inhibit neovascularization (Good et al., 1990; Iruela-Arispe et al., 1999). *In vivo*, TSP-1 peptides inhibit tumor angiogenesis (Miao et al., 2001). Data suggest the antiangiogenic activity is derived from its ability to induce endothelial apoptosis (Guo et al., 1997).

Although not as well studied TSP-2 contains sequences similar to those found in fragments responsible for the antiangiogenic activity of TSP-1. The molecule a whole

clearly has the ability to inhibit angiogenesis. TSP-2 also modulates MMP-2 activity which could indirectly function to inhibit angiogenesis by affecting endothelial cell adhesion and migration (Bornstein et al., 2000).

The C- terminal hompexin like domain, PEX, of MMP-2 has the ability to attenuate angiogenesis (Brooks et al., 1998). Its activity may rely on it interaction with $\alpha v\beta 3$ integrin thus blocking full length MMP-2 binding and a loss of proteolytic localization at the cell surface. PEX can be found enriched in regions of $\alpha v\beta 3$ expression, both in postnatal retinal neovascularization as well as in angiogenic areas of tumors at levels capable of inhibiting angiogenesis in various assays.

Angiostatin is another endogenous inhibitor of angiogenesis. This polypeptide is a fragment of plasminogen containing the four N-terminal triple loop disulfide-linked domains known as kringle domains (K1-4)(O'Reilly et al., 1994). The *in vivo* antiangiogenic activity of angiostatin resides in kringles 1–3, with kringle 1 being the most potent inhibitor and kringle 4 being relatively inactive (Cao et al., 1996). Its activity is to inhibit endothelial proliferation and migration and induce apoptosis. Angiostatin has also been reported to bind to $\alpha v\beta 3$ integrin which may have interesting implications for its mechanism of inhibition (Tarui et al., 2001).

The non-collagenase domains of collagens are proving to inhibit angiogenesis with varying effectiveness. The fragments can theoretically be generated endogenously by proteolytic cleavage of collagens yet none have been purified to date at physiologically relevant concentrations. Endostatin, the C-terminal NC11 domain of Collagen XVIII, is the prototype for this category of inhibitors (O'Reilly et al., 1997). It inhibits endothelial cell proliferation and tumor growth. Several studies have suggested

that it functions through binding to αvβ3 integrin (Rehn et al., 2001) although other groups are unable to reproduce this dependence (Kalluri, R., personal communication). Restin, the NC10 domain of Collagen XV, a close homolog of Collagen XVIII, also has exhibited antiangiogenic activity in xenograft tumor models (Ramchandran et al., 1999). In addition, several alpha chains of Type IV Collagen have been shown to inhibit angiogenesis. NC1 domains of α1, arrestin, α2, canstatin, and α3, tumstatin, have all exhibited antiangiogenic activity (Colorado et al., 2000; Kamphaus et al., 2000; Maeshima et al., 2000b).

In addition to proteolytic fragments of endogenous molecules, there are several complete endogenous molecules whose innate role is to inhibit angiogenesis. Again these molecules are being exploited as potential cancer treatments. TIMPS, tissue inhibitors of metalloproteinases, inhibit the remodeling of ECM and basement membrane. There are 4 family members but TIMP-1 and TIMP-2 have been best studied as inhibitors of MMP. TIMPs can inhibit the migration of endothelial cell *in vitro* and inhibit B16F0 tumor growth and angiogenesis *in vivo* (Brew et al., 2000). Expression of both TIMPs and MMPs is regulated by TGF-β and a very delicate balance between these inhibitors and their targets is achieved where any imbalance leads to disregulated angiogenesisIn addition, key regulators of vessel formation have been targeted to cause disruption and disregulation of angiogenesis through the synthesis of specific antibodies or peptidomimetics (reviewed in Klohs and Hamby, 1999). Once cross-reactivity problems were resolved, anti-VEGF reagents have shown reversible inhibition of active angiogenesis and are in Phase 3 clinical trials. The receptor has also been targeted by monoclonal antibodies and is undergoing preclinical trials. Several small molecule

inhibitors have been targeted to the tyrosine kinase activity of VEGFR-2 and thus inhibit downstream signaling events necessary to induce endothelial proliferation. Some are well developed and have shown selective inhibition and are promising cancer treatments. Vitaxin, an antibody against $\alpha v\beta 3$ integrin, has even been successful in early phase clinical trials at maintaining or shinking tumors (Eliceiri and Cheresh, 1999; Gutheil et al., 2000). Matrix metalloproteinases have also been targets of antibody and small molecule therapies. Inhibitors have been developed with increased specificity and have inhibited the growth and metastasis of several tumors and are currently in Phase 3 trials.

Just as these critical players can be interfered with to inhibit angiogenesis, they could be targeted in treatments needing increased vascularity. Recombinant growth factors appear most promising for the therapeutic induction of angiogenesis. In fact, human gene therapy for stimulating angiogenesis has been reported through gene transfer of VEGF or FGF gene in the treatment of ischemic myocardium or peripheral muscles and for the prevention of post-angioplasty restenosis because of its capability to induce endothelial repair and production of NO and prostacyclin. Early trials have successfully introduced the target gene and although they have passed Phase 1 trials with no side effects, did not show treatment effect as a single angiogenic factor (Epstein et al., 2001).

With all of the possible targets, one would hope a successful candidate (or candidates) could be identified for therapeutic intervention of angiogenic diseases, but then again because of all the possible targets it may be difficult to harness the complex system in the desired fashion. Despite all that is known and the treatments being developed, the process of angiogenesis and the exact role each component plays is still unknown. For most reagents he mechanism of inhibition is not known and until the

endogenous functions of these molecules is truly understood, the best treatments can not be actualized. The next section will describe the two molecules I have investigated hopefully to further understand their regulation of angiogenesis.

Thesis focus

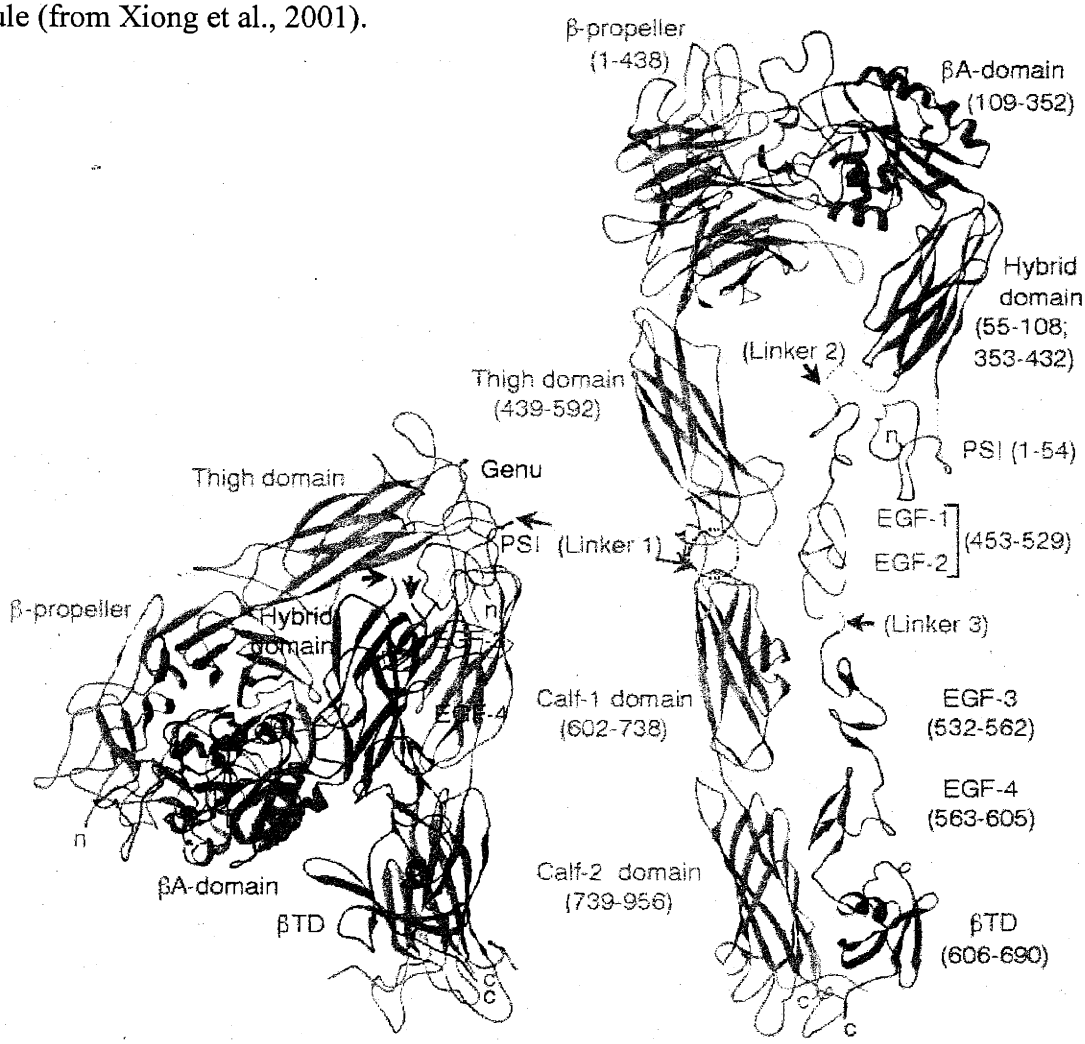
Integrin αvβ3 in Angiogenesis

Integrin αvβ3 has been implicated as a promoter of angiogenesis. This conclusion originated from localization data showing an upregulation of αvβ3 integrin on "active" endothelium, areas of growing vessels. The data appear clear that αvβ3 integrin expression is correlated with proliferation, migration and tubule formation. αvβ3 is a major vitronectin receptor but also binds several additional RGD-containing matrix molecules. This integrin can be found in focal contact and ECM adhesions. It can propagate survival, growth and migratory signals into the cell through focal adhesion kinase. In addition to matrix, αvβ3 has been shown to interact with integrin associated protein (CD47/IAP), VEGFR-2, MMP-2, PDGFR-β and Insulin receptor (reviewed invan der Flier and Sonnenberg, 2001). It has been generally accepted as well that in quiescent vessels, protein expression, in addition to mRNA expression, of αvβ3 integrin is downregulated. Recently, Singh et al. sought to clarify the expression patterns of the alpha v beta 3 -integrin in nonproliferating vascular beds (Singh et al., 2000). They examined organ-specific differences, and determined the constitutive presence of the alpha v and beta 3 mRNAs and the αvβ3 - heterodimer in the lung microvascular bed suggesting that gene transcription for the integrin is ongoing in the lung vessels. Expression is lacking in most other organs tested which raises the question if lung

endothelium is not actually quiescent or if some non-proliferating endothelium express αvβ3. The unique nature of this organ may account for the differences seen since demands for a more dynamic vasculature involved in the exchange of oxygen may be necessary for proper lung biology.

Figure 1.5: αvβ3 integrin

Recently the crystal structure of αvβ3 integrin was determined. Depicted here on the left is a ribbon drawing of the crystallized molecule and on the right a model of the extended molecule (from Xiong et al., 2001).



Prior to genetic ablation of αvβ3 integrin, much evidence regarding this integrin's functions arose from interference studies. Antibodies, peptides and other small

molecules have been used to perturb development of the vasculature in several of the models discussed earlier. Reagents specific for αvβ3 integrin have been developed and used to assay its function. LM609, a monoclonal antibody raised against human αvβ3 which cross-reacts with αvβ3 protein in many species (but not mouse), has been used as a competitive inhibitor of ligand binding when applied systemically in CAM and locally in corneal micropocket assays (Drake et al., 1995; Klotz et al., 2000). Likewise, cyclic RGD peptides, also binding to the ligand binding site, have been optimized for interaction with and specificity for αvβ3 integrin (Dechantsreiter et al., 1999; Kok et al., 2002; Pfaff et al., 1994). Experiments using these low molecular weight peptide and peptidomimetic inhibitors of αvβ3 integrin or antibody blockade in chick chorioallantoic membrane assay, corneal micropocket assay and tumor burden assays have shown that these antagonists of αvβ3 can function to inhibit angiogenesis.

Systemic administration of antagonists of αvβ3 ligand binding also have been shown to upregulate p53 DNA binding activity in proliferating endothelium in the chick CAM assay (Stromblad et al., 1996). This increase is accompanied by increasing $p21^{WAF1/CIP1}$ activity and blocking of bFGF-induced bcl-2 mRNA and protein expression on the CAM as measured by solution hybridization and Western blot analyses. When immobilized, antibodies act as agonists through their ability to cluster integrins on the cell surface, promoting cell adhesion and spreading (Leavesley et al., 1993; Stromblad et al., 1996). These reagents function as substitutes for vitronectin when immobilized but when added systemically or in solution, they argue that LM609, binding to the apical surface of the adherent cell, acts as an antagonist. Immobilized agonist mimics ligand binding in HUVECs and transmits cell survival signals by increasing the bcl2/bax ratio in

cells. Depending on the function being assayed these reagents act both as agonists and antagonist of that particular readout but not necessarily of the true endogenous function. This must be determined in order to understand how these reagents are working.

The promiscuity of αvβ3 binding increases with evidence that this integrin interacts extracellularly with another critical activator of angiogenesis, MMP-2. As discussed above, the degradation of basement membrane is critical for the initiation of endothelial cell migration. Brooks et al (1996) reports co-localization of gelatinase A (MMP-2) and αvβ3 on the surface of melanoma cells and angiogenic blood vessels. Logically following this discovery, PEX, a proteolytic fragment of MMP-2, mentioned above as an inhibitor of angiogenesis, was found to interfere with the interaction of αvβ3 and MMP-2 and thus inhibit angiogenesis (Brooks et al., 1998).

Incongruous with the interpretation of experiments using β3 antagonists, animals genetically deficient for αvβ3 have no gross defects in vasculogenesis or normal angiogenesis. The targeted disruption of the αv integrin subunit by Bernhard Bader and the β3 integrin subunit by Kairbaan Hodivala-Dilke in our lab did not lead to overlapping phenotypes. As I mentioned earlier, the αv-null animals develop extensive vasculature only to suffer from brain hemorrhaging which leads to their death whereas the β3-null animals survive with minor complications primarily associated with the loss of αiibβ3 integrin on platelets. Interestingly the additional absence of theβ5 integrin subunit a closely related αv partner expressed on endothelial cells does not lead to any substantial defects suggesting that αvβ3 and αvβ5 are not essential for developmental angiogenesis.

Conflicting reports more recently suggested that this integrin independently inhibits as well as promotes angiogenesis. Tumor studies in β3-/-, β5-/- or animals which

lack both β3 and β5, all indicate that these integrins are not essential for pathological angiogenesis. Our group has shown however that it is affected in these animals. Pathological angiogenesis is enhanced by the lack of αvβ3 (or αvβ5, or both β3 and β5), not suppressed as would be predicted from the inhibitor studies (Reynolds et al., 2002). In several subcutaneous tumor growth models (B16, CMT19T, LS180 and A375 tumor cell lines), growth is enhanced in animals lacking beta 3 integrin. Unlike previous inhibitor studies, in hypoxia-induced tube formation assays, Kroon et al (2000) was unable to see any inhibitory role of LM609 antagonist of αvβ3 integrin.

In addition to PEX, several antiangiogenic factors have been shown to bind αvβ3, although the significance of these interactions is not yet certain. It is necessary to determine if this binding endogenously occurs and if the functional interaction interferes with normal αvβ3 activity or activates a novel antiangiogenic activity of this receptor. In addition to heterodimerizing with β3, the alpha v integrin subunit also partners with beta 5 in endothelial cells and much effort has been exerted to distinguish the functions of these integrins in angiogenesis. Friedlander et al first proposed a mutually exclusive role for beta 3 or beta 5 integrin in growth-factor mediated stimulation of endothelial cells (Friedlander et al., 1995). Testing *in vivo* angiogenesis in corneal or chorioallantoic membrane models, they reported that addition of antibodies specific to either αvβ3 or αvβ5 integrin blocked only bFGF- or VEGF-induced angiogenesis, respectively. They conclude from their data, which will be discussed in greater detail in Chapter 3, that αvβ3 integrin function therefore was necessary for mitogenic stimulation by bFGF whereas VEGF stimulation required the function of αvβ5 integrin. These data have long stood as the model of the difference between each of the endothelial alpha v integrins.

Unfortunately, this theory is complicated by the paucity of defects in the αvβ3 / αvβ5 double knockout animals. According to this theory there would be no VEGF or bFGF mitogenic signaling in the endothelium of these animals and one would have to wonder how they are viable and fertile.

Although the number of papers suggesting that αvβ3 may function as an antagonist of angiogenesis are few the quality of the data is strong. Past results could potentially be analyzed in light of recent data to be consistent with this theory. There is certainly not sufficient data at this point to rule out either a proangiogenic or an antiangiogenic role or both for αvβ3. Alternate theories on the endogenous role of αvβ3 incorporating data presented in this thesis will be discussed in Chapter 5.

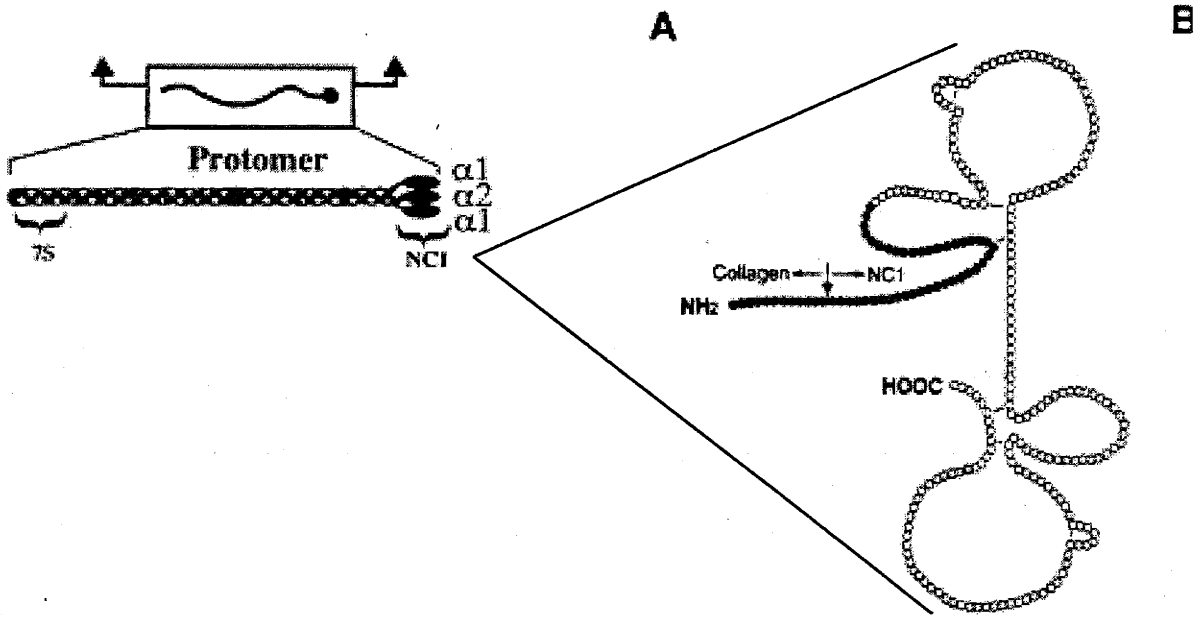
Type IV Collagen

Basement membranes are organized thin layers of specialized extracellular matrix that provide the supporting scaffold for epithelial and endothelial cells (Paulsson, 1992). Basement membranes not only provide a mechanical support but also influence cellular behavior such as proliferation, migration, and differentiation of various cells including endothelial cells. Vascular basement membrane is speculated to play an important role in regulating pro- and anti-angiogenic events (Colorado et al., 2000; Kalluri and Sukhatme, 2000; Madri, 1997). Type IV collagen is the major collagenous component found in an established basement membrane (Timpl, 1996). The structure of Collagen IV family consists of a globular C-terminal non-collagenous domain (NC), a central triple helical domain and a cysteine rich N-terminus. Type IV collagen is thought to be enhance endothelial cell proliferation and influence behavior during the angiogenic process

(Colorado et al., 2000; Madri, 1997; Maeshima et al., 2001a). There are six distinct α-chains, α1 - α6, which make up the Type IV collagen family (Prockop and Kivirikko, 1995). These α-chains assemble heterotypically into triple helices that further network into a scaffold onto which additional macromolecules bind to form the complex basement membrane. Each α-chain is composed of three domains, the N-terminal 7 S domain, the central helical domain, and the C-terminal globular noncollagenous domain (NC1) (Timpl et al., 1981). The NC1 domain of type IV collagen plays a crucial role in the assembly of type IV collagen to form trimers and subsequent networks and thus influences basement membrane organization, which is important for new blood vessel formation (Madri, 1997; Timpl, 1996; Tsilibary et al., 1990).

Figure 1.6: Schematic of Type IV Collagen and NC1 domain structure

Type IV Collagen exists as a triple helical molecule whose subunits associate through their C-terminal non-collagenase domains (NC1). The NC1 domain of α3 Coll IV contains the anti-angiogenic activity and its structure is expanded in (B).



Reports generally conclude that α1 and α2 IV Coll are the predominant isoforms found in vascular basement membrane, while other isoforms are present in more specialized basement membrane. Recent data however suggests a more widespread expression pattern of various alpha chains (personal communication, Kalluri, R.). Tumstatin, the subject of Chapter 4, is generated by cleavage of the NC1 domain of α3 (IV) Collagen. It has been shown by collaborators to inhibit tumor growth in several models (Maeshima et al., 2000b). Targeted disruption of α3 (IV) collagen resulted in

viable mice mimicking the human disease progression of autosomal Alport Syndrome, a progressive glomerulanephritis disease with varying ear and eye complications (Cosgrove et al., 1996; Miner and Sanes, 1996). Additional disruption of α4 (IV) resulting in a double knockout, generates animals with similar phenotypes but an earlier onset of disease (Lu et al., 1999). Mutations in α3, α4 and α5 have been reported in humans manifesting Alport Syndrome (Lemmink et al., 1994; Mochizuki et al., 1994). Analysis of patients indicated an interdependence of protein expression of these three alpha chains; that is in the absence of any one chain, no other chain is incorporated into glomerula basement membrane although this is not a hard and fast rule as data from α3(IV) deficient mice suggests that in the ear and eye α5(IV) incorporation into BM is not dependent on α3 expression (Kalluri and Cosgrove, 2000). Preliminary data in the α3 (IV) collagen knockout animals suggests the absence presumably of the α3(IV) NC1 cleavage product promotes increased tumor growth in these animals (Sugimoto, H, personal communication).

Thesis Perspective and Plan

My original thesis proposal outlined my plans to investigate the *in vitro* effects of selectin deficiency in endothelial cells. Selectins are implicated in the extravasation of leukocytes and possibly metastatic tumor cells. There are 3 family members, E-, P- and L- selectin, two of which, E- and P-, are expressed on endothelium. P-selectin is constitutively translated, stored in Weibel-Palade bodies and translocated to the plasma membrane within 10 minutes of histamine and thrombin treatment. E-Selectin, as well as P-Selectin, can be upregulated transcriptionally and directly transported to the cell

surface in an NFκB-dependent manner in endothelial cells. L-selectin, on the other hand, is expressed on the microvilli tips of activated neutrophils and other circulating leukocytes. Ligands for selectins are expressed on the respective "opposing" cell. The interaction of selectins and their ligands wis thought to be responsible for the physical tethering of cells to the endothelium and I wanted to investigate if there were any additional functions of these interactions.

When I commenced my thesis, single knockout strains for each selectin were available as well as the P/E double knockout while the other double knockouts and triple knockout were being generated in the lab (Robinson et al., 1999). In addition, targeted disruption of ICAM-1 (Xu et al., 1994), β2 integrin (Wilson et al., 1993) and specifically αmβ2 integrin (Coxon et al., 1996), molecules also involved in transendothelial migrations, were available. My aim was to develop procedures for purifying EC from these strains, establish *in vitro* transmigration assays to investigate defects in this process and determine the roles of individual selectins and their ligands. I worked on EC purification methods while establishing transmigration assays with HUVECs and other established endothelial cell lines. The latter, transmigration, proved more difficult to study than I had hoped and other developments in the field rendered the analysis of transmigration assays less appealing. I did, however, develop reliable methods for isolating EC, initially using the selectin knockouts. Developments in angiogenesis studies that I have reviewed above raised interesting questions which seemed a more productive arena in which to use my endothelial cell preparations.

In this thesis, I will describe to you and provide evidence of a reproducible and reliable method of isolating and characterizing primary murine lung endothelial cells

(MLEC) (Chapter 2). In chapter 3, I will discuss the analysis of angiogenic responses of β3-deficient animals and cells in comparison to the wild-type processes, providing evidence which will begin to suggest a novel role for αvβ3 integrin in blood vessel development. I will then discuss the analysis of a relatively new member of a growing class of angiogenesis inhibitors, tumstatin (Chapter 4). I will apply these purified MLEC in the analysis of tumstatin activity and provide proof of the **necessity for** but not the sufficiency of β3 integrin for tumstatin function. In addition, I will confirm the necessity for beta 3 integrin by providing data of *in vivo* angiogenesis assays. I will present results to support the beginnings of a basic mechanism of tumstatin, which I will discuss in Chapter 4.

The data I present in the following pages of my thesis will support testable theories of the mechanism by which tumstatin can inhibit angiogenesis and these theories will provide insight into a novel model which begins to clarify the role of αvβ3 integrin in angiogenesis.

Chapter 2: Isolation and Characterization of Primary Mouse Lung Endothelial Cells

Section 2.1: Introduction

At the beginning of my studies there were few well-established primary endothelial cell lines and none characterized from mouse tissue (see Introduction for detail). Two limitations existed in the supply of well-defined cell-lines: availability of source material and relevance to current investigations, including available bioreagents. HUVECs (human umbilical vein endothelial cells) were used as the laboratory standard in assays of endothelial function. Assays, in fact, were developed for these cells and subsequently adapted as newly emerging cell lines appeared. The limited proliferative capacity of HUVECs (5-7 passages) however, required frequent isolation from human tissue of variable origin. HUVECs also represented a single vascular bed and a large vessel at that. Capillary endothelial cells from bovine origin (BCEC) as well as large vessel bovine aortic endothelium (BAEC) were both isolated and used in many of the same assays. Bovine endothelial cells have been isolated since the 1970's due to the abundant source of these cells obviously with decent yield and less concern about blood-borne pathogens carried in human tissue culture. These cells, also classified as primary endothelial cells, have a much greater proliferative capacity (15-20 passages). Each primary cell line is from a distinct vascular bed and possesses distinctive characteristics (Auerbach et al., 1985; Sage et al., 1981; Schor and Schor, 1986). Bovine cells have proven useful in biochemical assays in which large amounts of material are necessary such as purification of novel molecules and studies on endothelial anion channel function and Ca++ flux (McPherson et al., 1981; Sedova and Blatter, 1999). Yet there are many

fewer bovine-specific reagents such as antibodies and recombinant cytokines available compared to the many developed for use with human cell lines.

Methods for isolating endothelial cells from murine tissue, on the other hand, had only just begun. Purification methods were tedious and yield was lower than desired. Additionally, selection methods often were not specific enough to eliminate contaminating macrophages or other cell types and reagents directed to murine endothelial-specific antigens were just being developed (Gumkowski et al., 1987). Murine cerebral endothelial cells were in fact the only primary microvascular cell line known to our lab (Barkalow et al., 1996), and originated from a highly specialized vascular bed. In addition, cells from high endothelial venules (HEVs), specialized postcapillary venules, found in lymphoid organs and chronically inflamed tissues were also being studied (Girard et al., 1999). However, difficulties in their purification and in vitro maintenance hampered their use in studies. An adaption of an *in vitro* differentiation method used to isolate hematopoeitic lineages provided a method for differentiation of murine embryonic stem cells into endothelial cells (Vittet et al., 1996). The yield of a purified population is extremely low for this method but it does provide an in vitro method for analysis of cells nullizygous for various molecules unable to generate viable animals (Francis et al., 2002; Taverna and Hynes, 2001).

A reliable method for isolating endothelial cells from mice would provide an indispensable reagent for many critical assays. Specific reagents such as antibodies, growth factors and cytokines have been developed for use with murine tissues and cells. The ability to purify cells from viable animals provides the opportunity to purify and assay endothelial cells with genetic alterations; transgenic animals and animals deficient

for molecules of interest can be used as sources of endothelial cells. These cells can then be used to assay specific effects due to the genetic alteration on endothelial function.

Endothelial cells can vary greatly depending on the vascular bed from which they are isolated. Cerebral endothelial cells possess many characteristics unique to the blood-brain barrier. Although many of these properties are dependent on the microenvironment and associated peripheral cells, for example pericytes or astrocytes, interacting with the endothelium (Hayashi et al., 1997), certain characteristics are maintained in culture which are specific to this cell type (Arthur et al., 1987; Gaillard et al., 2001; Janzer and Raff, 1987). My initial interests in inflammation and the transmigration of cells across the endothelial barrier led to the decision to purify lung endothelial cells. Lungs are accessible, have few contaminating cell types, can be perfused easily to eliminate red blood cell contamination and are primarily microvascular. The body of literature dealing with inflammation in the lung and associated disease was very large (Bevilacqua et al., 1994; Laitinen and Laitinen, 1994; Pilewski and Albelda, 1993), making this tissue a promising source of endothelial cells which presumably would maintain a highly responsive phenotype to inflammatory cytokines. Methods to purify endothelium from bovine lungs had already proven to yield a nice microvascular cell line (C-PAE) (Jones, 1979).

Our lab's particular interest in adhesion molecules and the availability of many viable animals deficient in these molecules is an ideal experimental situation for the use of primary mouse endothelial cells. Due to the complexity of studying many endothelial cell functions in vivo, yet the desire to examine more closely these functions in the absence of targeted adhesion molecules, my goal was to develop a reproducible method

for isolating microvascular murine cell lines which could be used to study in vitro endothelial cell function. The sole published isolation method for a murine cell line at inception of my work in this field was by Mary Gerritsen with whom I was able to speak (Gerritsen et al., 1995). Despite the publication of the method, she warned me of many difficulties and recommended I speak with a co-author, Bill Atkinson, a technician working nearly full-time on cell isolation and maintainence. The two had been working together on improving methods for isolation of mouse endothelial cells. I was able to get a method of harvesting tissue based on their work as well as several ideas from both on purification schema. I am certainly indebted to this initial help in generating the method which I present and analyze in this chapter. The purification methods that will be described herein represent several years of work and fine tuning to achieve a highly reproducible and reliable cell culture system.

Section 2.2: Results

Mouse Lung Endothelial Cell Culture

A population of endothelial cells isolated from the lungs of mice have been isolated and purified based on expression of ICAM-2 adhesion receptor as outlined below (Reagents information in Methods).

For 3 mice of same genotype - 8-13 wk. of age (have tried 2.5-20wk old successfully)

Day 0:

1. Sacrifice 1 mouse via cervical dislocation (not CO_2 or avertin)
2. Clean mouse with ethanol (do not get into mouth/lungs)
3. Open chest cavity (above diaphragm)
4. Perfuse lungs with PBS-heparin (1U/ml) (≈8ml, cold)[1]
   Inject right ventricle/cut left atrium
5. Collect Lung/heart into cold Ham's F-12 (20ml in 50 ml tube)
6. Repeat for each mouse

Move to tissue culture hood

7. Open 2 10cm tissue culture dishes
   a. In one dish aliquot 10ml 70% ethanol
   b. In other dish aliquot 10ml medium
8. In one petri dish lid pour out lungs and Ham's F-12
9. Dissect lungs, one lobe at a time, away from heart, dip in ethanol briefly and then place in medium.
10. Once all lung tissue has been collected into medium, move all tissue pieces into dry tissue culture dish lid.
11. Mince into very small pieces (patè/mousse consistency)
12. Transfer minced tissue to 50ml tube
13. Digest 1 hr. in 15ml, 0.1% collagenase at 37°C
    Shake occasionally (tissue usually settles as it digests)

Back in hood

---

[1] Perfusion is not the most important step, if it does not go well. continue anyway. If too much pressure is applied, lung capillaries may burst and perfusion will not go well.

14. Homogenize digested tissue by passing through 14g cannula (5-10 full passages) until large pieces are eliminated. Some white (fatty-like) pieces may remain. Do not overhomogenize.[2]
15. Filter through 140μm sieve into t.c. dish. Save unfiltered tissue.
16. Collect filtrate into 50ml tube with 20ml medium.
17. Spin down; 1200rpm (under table Beckman), 4°C, 5min.
18. Collect supernatant and add to unfiltered tissue (from step 15). Caution: Pellet may not be very well packed
19. Repeat homogenization (step 14-17) with unfiltered tissue and supernatant,
20. Aspirate supernatant. Caution: Pellet may not be very well packed.
21. Resuspend pellets in 12ml medium and plate into 2, 0.1% gelatin-coated T75 flasks.
22. 3-16h later, plates are washed well (3-5 times) with PBS or HBS to remove any rbc, non- or loosely-adherent cells.

Antibody Coated beads preparation (Per T75):

Negative Selection Beads**:
10 μl Rat anti-mouse FcγRII/III (Pharmingen-01241D)
500 μl PBS/2%FBS
50 μl Dynabeads M-450 Sheep anti-Rat IgG (Dynal)

Positive Selection Beads**:
10 μl Rat anti-mouse ICAM-2 (Pharmingen-01800D)
500 μl PBS/2%FBS
50 μl Dynabeads M-450 Sheep anti-Rat IgG (Dynal)

Incubate overnight @ 4°C to conjugate specific antibody
Before using, wash 3x in PBS/ 2% FBS
place beads in magnet,
aspirate supernatant,
remove from magnet
resuspend beads in 8ml PBS/FBS
repeat 2x
Aspirate PBS/FBS
Resuspend beads in 1.5ml medium

**Can be stored 6mo. @ 4°C, per manufacturer

Day 2:

1. Add 3ml medium to each flask of cells and place at 4°C to cool (10min-1hr)
2. FcγRII/III Antibody Coated Beads (in Medium (1.5-3ml)) added to each dish
3. Incubate 4°C 1hr.

[2] If overhomogenize, pellet is never well-packed, may not even have pellet.

4. Shake occasionally
5. Trypsinize cells
6. Resuspend in medium and place into 15ml tube in magnet
7. Collect supernatant
8. Replate into equivalent number of 0.1% gelatin-coated T75's (ie if originally plated in 2 dishes, keep in 2 dishes, if plated in 1 keep in 1 dish)

Day 4

1. Dishes washed
2. 3ml medium added and dishes placed at 4°C to cool (10min-1hr)
3. ICAM-2 Antibody Coated Beads (in Medium (1.5-3ml)) added to each dish
4. Incubate 4°C 1hr.
5. Shake occasionally
6. Trypsinize cells
7. Resuspend in medium and place into 15ml tube in magnet
8. Discard supernatant
9. Rinse beads with medium
10. Place in magnet
11. Discard supernatant
12. Resuspend beads in 12ml medium
13. Replate into equivalent number of 0.1%gelatin coated T75's (ie if originally plated in 2 keep in 2 dishes, if plated in 1 keep in 1 dish)

Another Postive Selection done somewhere between Day 5-8 (at 70-80% confluency) depending on growth of cells. At this sort, dishes can be split 1:2.

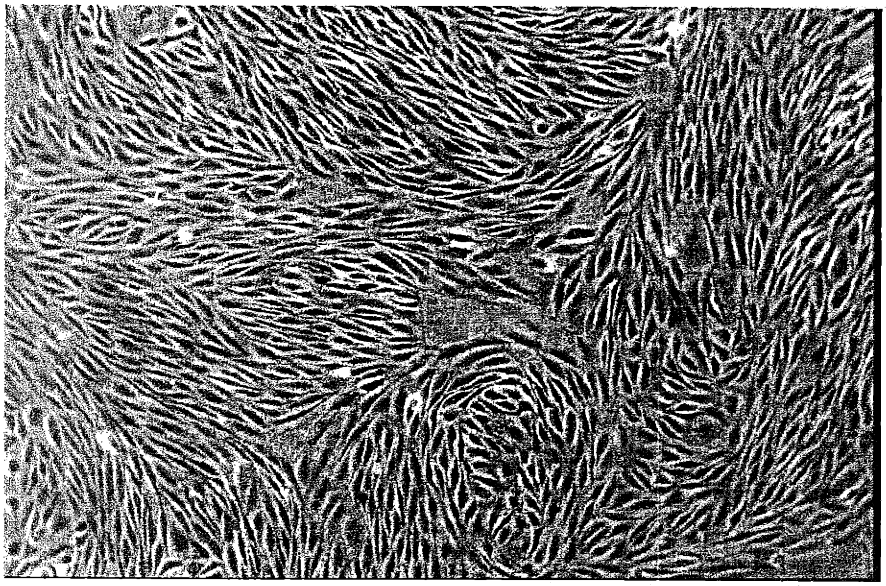
Primary cultures of enzymatically and mechanically digested mouse lungs contained endothelial cells, fibroblasts, macrophages and lung epithelium. Subsequent magnetic sorting depleted FcγRII-expressing cells, primarily macrophages. The FcγRII-negative population was expanded and a second magnetic sort selected ICAM-2 positive cells yielding a highly enriched endothelial population. Dynabeads coated with anti-ICAM-2 antibody efficiently bound a large majority of cells with endothelial morphology. Greater than 90% of cells which have been sorted as positive for ICAM-2, and as many as 97%, exhibited a clustered, cobblestone morphology typical of an endothelial cell culture. This apparent purity (by morphology) has been verified by addition of anti-ICAM-2 conjugated beads and visual observation of the percentage of

cells bound by beads. This second sorting for cells expressing ICAM-2 generated a population of mouse lung endothelial cells >99% pure (Fig 2.1). This homogeneous population maintained a characteristic endothelial morphology for up to 5 additional passages.

Magnetic beads were incorporated into MLEC but had no apparent effect on cell proliferation or function at low bead to cell ratio (< 10:1). Cells which bound and incorporated greater than 10 beads were less capable to proliferate as judged by the lack of dilution of beads despite overall increase in cell number, whereas cells with beads present in lower density (<10 beads/cell) continued to divide and the beads were diluted out in the resulting population.

Figure 2.1: Morphology of cultured Mouse Lung Endothelial Cells

Passage 4 wild-type mouse lung endothelial cells. Heterogeneous population isolated from the mouse lungs was negatively sorted to remove cells expressing FcgRII and positively sorted to enrich for cells expressing ICAM-2.



General Characterization

The endothelial population was identified at multiple steps of the purification as cells that bound and endocytosed acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-1,3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) (Fig. 2.2). Endothelial cells express a scavenger receptor involved in Ac-LDL uptake and metabolism. Voyta et al.(1984) showed an increase of 7-15 times more [^{125}I]-Ac-LDL internalized in endothelial cells over smooth muscle cells or pericytes and that this trait was an effective way of identifying this cell population.

The cells of the lung of one animal, plated into one, 75cm^2 flask on the day of isolation and sorted as suggested above yielded approximately a confluent 75cm^2 dish of passage 3 mouse lung endothelial cells (>99% pure) in 10-14 days. To extrapolate back, based on a doubling time of approximately 36 hours and a plating efficiency of ≈50%, the original endothelial population was probably roughly 0.5-1 x10^4 cells. These cells could be expanded at ≈80% confluency, into three, 75cm^2 dishes from one dish. In theory, the cells could be expanded in this fashion 2 or 3 times, for a total yield of on average of 25 confluent 75 cm^2 dishes, each roughly 2-5 x 10^6 cells. Although I never completely expanded a single preparation, since earlier passage primary cells are preferable, the total cell yield would have reached 1x10^8 endothelial cells per mouse.

Figure 2.2: DiI Acetylated LDL uptake

Passage 3 wild-type (A) and beta 3 integrin-deficient (B) mouse lung endothelial cells incubated with diI Ac LDL for 24 hours. Both cell lines express scavenger receptors specific to endothelial cells which bind and endocytose this fluorescent marker.

A.

B.

Variations

Alternative strategies for obtaining a pure endothelial population included varying order and number of magnetic bead selections. These variations were performed based on the morphological appearance of the heterogeneous population as well as efficiency of selection. When a very high yield was critical, the population of cells that did not bind anti-ICAM-2 conjugated Dynabeads (Day 4, Step 11), including a minor population of cells with the morphological appearance of endothelial cells, was re-plated independent of ICAM-2-positive cells. These cells were expanded and retained their endothelial morphology. ICAM-2 became expressed on the surface of these cells after a single passage and could be used to enrich this previously ICAM-2-negative population for endothelial cells. On the occasion there were few macrophage looking cells and a large initial percentage of endothelial looking cells, my preparation would proceed directly with ICAM-2 positive selection (Day 4, 1)with yields as high as 98% pure endothelial cells judged by cell morphology. The number of positive selections could also be varied as judged by contaminating cell populations until a highly purified endothelial cell population was achieved. Regardless of variations in sorting a final ICAM-2 positive selection yields a morphologically homogeneous endothelial population.

I isolated and purified MLEC from several viable transgenic animals developed in the Hynes Lab as well as non-transgenic control mice. The selectin family of molecules consists of 3 members, E-, P- and L- selectin. E- and P- selectin expressed on endothelial cells along with L-selectin expressed on leukocytes, play a role in tethering circulating cells to the vascular walls. Endothelial cells deficient in E-selectin or P-selectin or both

selectins were harvested and characterized. These cells grossly appear to behave identically, sans selectin expression, to wild-type cells in routine culturing. In addition, cells were harvested from animals lacking beta 3 integrin, or both β3 and β5 vascular integrins, which will be discussed further in other chapters.

Immunoreactive Profile/Characterization of MLEC

To validate the endothelial nature and purity of the final cell population, this population was assayed for expression of characteristic markers common to endothelial cells (Garlanda and Dejana, 1997). For all antigens tested a fair amount of information is known about the protein function in endothelial cells and will be discussed in some detail. Unfortunately, many EC markers are also expressed on hematopoietic cells complicating purification schemes and requiring multiple markers to confirm cell identity.

von Willebrand Factor (Factor VIII related antigen) has multiple ligands in the vasculature. It mediates platelet aggregation and thrombus formation. VWF is expressed constitutively and stored in both the α-granules of platelets and in Weibel-Palade bodies in endothelial cells, and is rapidly mobilized and secreted in response to histamine. Ligands include collagen, αIIbβ3, and GP Ibα. A percentage of the isolated primary endothelial cells stained positive for vWF, a commonly used marker for endothelial cells at early passages only (Fig.2.3). This staining is lost with subsequent passaging of MLEC as has been observed for many cultured endothelial cells.

Figure 2.3 vonWillebrand Factor staining of MLEC

A. A heterogeneous first passage population of purified cells of the lung including endothelial cells stained for von Willebrand Factor. Punctate intracellular staining is seen in the endothelial cell subpopulation.

B. Passage 3 MLEC occasionally express vWF but much less intensely than in cells in which staining can be seen at first passage.

A.

B.

Platelet Endothelial Cell Adhesion Molecule (PECAM-1/CD31/EndoCAM) is a 130kDa adhesion molecule in the immunoglobulin supergene family (IgSF) that is expressed on endothelial cells, platelets, and some hematopoietic lineage cells (Newman et al., 1990). This molecule has been further defined to a subfamily characterized by the presence of one or more immunoreceptor tyrosine–based inhibitory motifs (ITIMs) within their cytoplasmic domains (Vivier and Daeron, 1997). This molecule is enriched at intercellular junctions of endothelial cells (Muller et al., 1989). Homophilic PECAM-1 adhesion occurs through direct interaction of only the 1st and 2nd of 6 extracellular Ig-homology domains; Fab antibody fragment interference with homology domain 6 in fact augments activity of PECAM-1 (Sun et al., 1996a; Sun et al., 1996b). Evidence for heterotypic trans-interaction of PECAM-1 with alpha v beta 3 integrin may be true for the murine molecules (Buckley et al., 1996; Piali et al., 1995) but similar binding studies with human PECAM-1 have not supported this theory (Sun et al., 1996b). Wong et al., (2000) further characterized the murine heterotypic interaction and showed no binding at intercellular junctions but showed that PECAM-1 and $\alpha v\beta 3$ may interact laterally in the same cell in an RGD- independent fashion in cocapping experiments. Immunofluorescent staining of MLEC with antibody to PECAM-1 (CD31) showed overall cell surface expression with a concentration at cell-cell contacts as expected (Fig. 2.4). Fluorescence-activated cell sorting (FACS) of non-permeabilized cells indicates that the entire population of MLEC express PECAM-1 on the cell surface (Fig. 2.4).

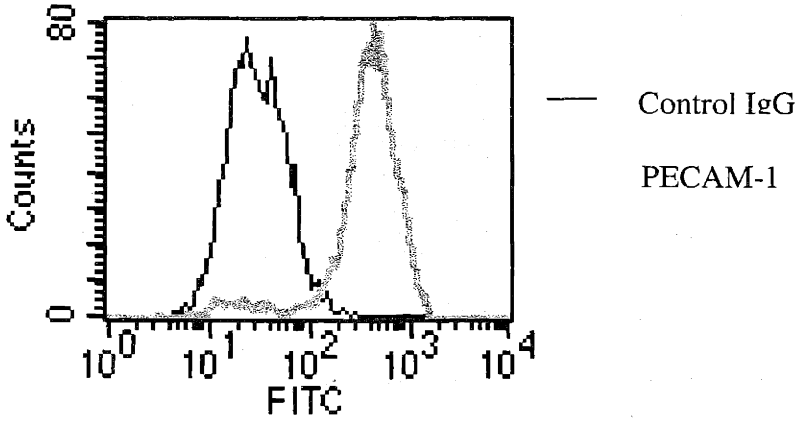
Figure 2.4 PECAM-1 Immunolocalization and Quantitation.

A. PECAM-1, visualized with rhodamine conjugated secondary antibody, is localized at intracellular junctions of confluent Passage 4 MLEC.

B. FACS plot of MLEC incubated with PECAM-1 antibody. The entire population of cells is reactive with PECAM-1 ( ) antibody as indicated by a complete shift from control IgG plot (-).

A.

B.



The Intercellular Adhesion Molecule (ICAM) family has five structurally related immunoglobulin superfamily members identified. ICAM-1 is by far the best characterized member and can be expressed on endothelial cells along with ICAM-2. ICAM-1, unlike ICAM-2, is expressed a very low levels on resting endothelium but its expression can be induced by inflammatory cytokines including TNF-α, IL-1β and IFN-γ. ICAMs participate in cell:cell adhesion through β2 integrins expressed on leukocytes. ICAM-2 is reported to be expressed constitutively on endothelial cells as well as on some hematopoietic lineages including monocytes, platelets and lymphocytes (Nortamo et al., 1991). Several reports indicate that ICAM-2 expression can in fact be downregulated in cytokine-stimulated endothelium (Langley et al., 1999; McLaughlin et al., 1998; Silverman et al., 2001). MLEC express ICAM-1 strongly in both a punctate staining pattern and at the cell periphery (Fig 2.5 A). Cells obviously express ICAM-2, as this antigen was used to purify the population. Its pattern is more diffuse than ICAM-1 (Fig 2.5 B).

Endothelial cells express another related member of the immunoglobulin superfamily, Vascular Cellular Adhesion Molecule-1 (VCAM-1). VCAM-1, like ICAM-1 is regulated by inflammatory cytokines and bacterial endotoxin (LPS) (Hauser et al., 1993). VCAM-1 is involved in preliminary steps of transendothelial migration. VCAM–1 binds α4β1 (VLA-4) expressed on leukocytes. VCAM-1 is expressed in a similar pattern to ICAM-1 on MLEC including both punctate surface expression as well as an accumulation at the cell periphery (Fig 2.5 C).

Figure 2.5 Immunofluorescence Staining for Endothelial Markers

Passage 3-4 wild type MLEC were stained with the indicated primary antibody and FITC-conjugated secondary antibody. Samples in A and C. were treated for 4 h with TNF-a (10u/ml) to induce antigen expression.

A. ICAM-1

B. ICAM-2

C. VCAM-1

The dynamic nature of the vascular microenvironment requires endothelial cells to be highly responsive and in respect to conditions of inflammation, this manifests itself by the ability to increase the surface expression of many adhesion molecules (Hahne et al., 1993). In order to assess whether MLEC maintained this responsive phenotype in culture, cells were assayed by FACS analysis for their ability to upregulate surface expression of NFkB-inducible endothelial markers in response to LPS (1 μg/ml) and TNF-α (10U/ml) stimulation. Representative histograms of ICAM-1, VCAM-1 and E-Selectin show a shift in mean intensity that was seen in FACS analysis of inducible proteins. (Fig. 2.6) Populations were gated and the percentage of fluorescently labeled cells falling into the gate were determined. Table 1 presents the quantification of FACS analysis of several inducible molecules and the profile of induction of these markers in MLEC is comparable to the profile seen in other endothelial cell lines (Haraldsen et al., 1996). Analysis of E-L-P- selectin triply deficient animals confirms the specificity of the upregulation of selectins on the cell surface of MLEC. Unexpected constitutive high expression of ICAM-1 was common yet there was still a detectable shift upon stimulation with mediators of inflammation.

Figure 2.6: Inducibility of Surface Expression of Endothelial Markers on MLEC.

After 4 hours of induction (TNF-α ; LPS; or no treatment) FACS analysis with the indicated antibody was performed on trypsinized cells as described in the Methods section 6.4. Representative plots are shown of wild-type MLEC. There is a clear shift of surface expression seen with cytokine treatment, and the percent of cells falling into a gated positive region are listed in Table 2.1.

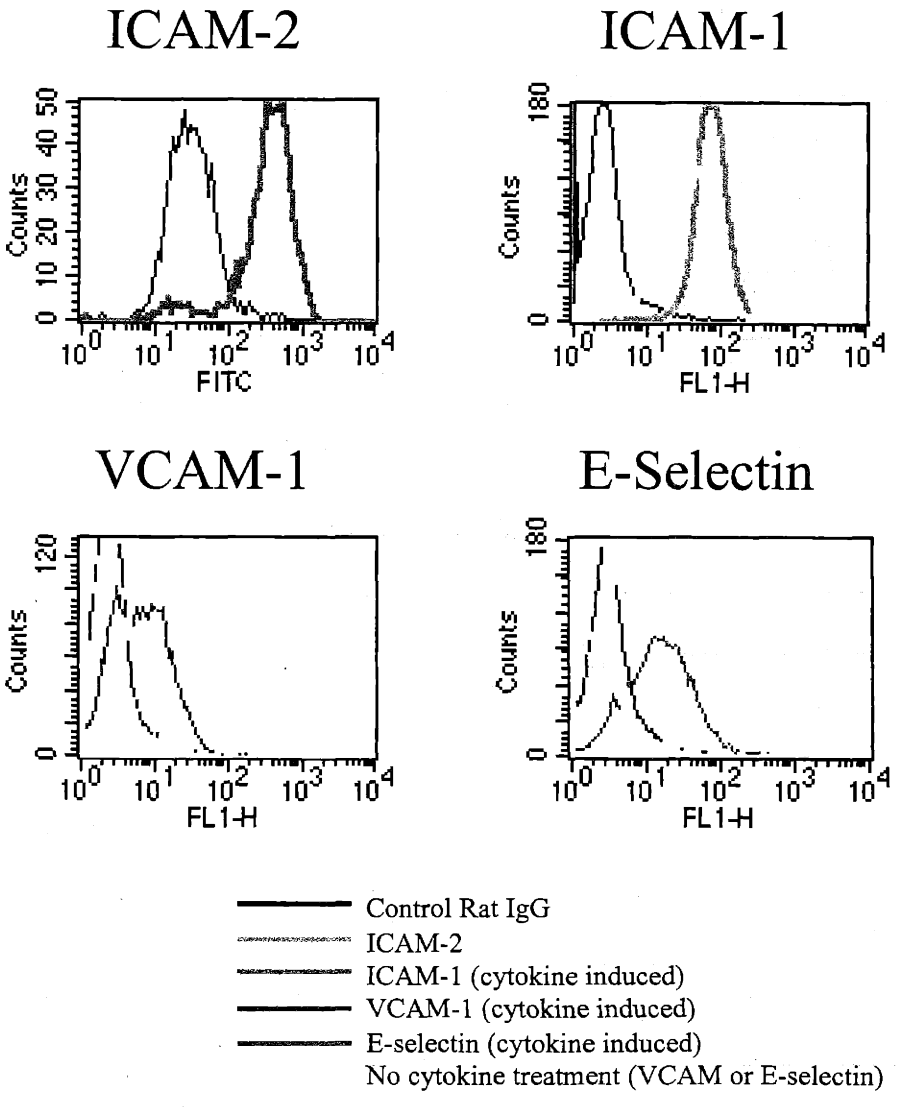


Table 2.1: Quantification of FACS Analysis of Endothelial Markers

MLEC were analyzed by FACS analysis as outlined in Method 6.4. A shift in mean fluorescent intensity indicated a positive antibody reaction. MLEC were analyzed without (Uninduced) or with (Induced) stimulation for 4 hours with LPS (1 μg/ml) and TNF-α (10U/ml) at 37°C. All markers tested exhibited expected upregulation of surface expression following this stimulation with the exception of ICAM–1. The number of cells with reactivity to the indicated antibody above control IgG reactivity is indicated as a percentage of all cells analyzed. ICAM-1 was expressed constitutively on nearly all MLEC and therefore further upregulation could be assessed by a mild shift in mean intensity only.

	wild type MLEC	
	Uninduced	Induced
PECAM-1	70%	98%
ICAM-2	99.9%	99.9%
ICAM-1	95%	99.9%
VCAM-1	4%	40%
E-Selectin	5%	75%
P- Selectin	6%	45%
FcγRII	3%	3%

	ELP-/- MLEC	
	Uninduced	Induced
PECAM-1	98.8%	89%
ICAM-2	99.9%	99.9%
ICAM-1	87%	99.5%
VCAM-1	6%	35%
E- Selectin	6.6%	3%
P- Selectin	3%	3%
FcγRII	6%	3%

Characterization of adherens junction formation

A key component of cell-cell adhesions is an endothelial-specific member of the cadherin family, VE-cadherin (cadherin-5) (Lampugnani et al., 1992). A transmembrane molecule, which participates in homophilic calcium-dependent interaction with adjacent cells, plays an important role in vascular permeability and integrity (Dejana et al., 1999). Expression of endothelial-specific VE-cadherin, along with associated molecules alpha-catenin and beta-catenin, was concentrated at intercellular junctions by immunofluorescent staining of MLEC (Fig 2.7). Association of intracellular catenin molecules with the cytoplasmic tail of VE-cadherin initiates changes in cell morphology through interactions with the cytoskeleton component. To determine the time after plating at which MLEC form a confluent monolayer with functional intracellular junctions, a time-course immunofluorescence analysis of adherens junction proteins was conducted. In transendothelial migration assays it is critical to form a functional cellular barrier through which cells must initiate an active process to pass. In addition to visible gaps, I wanted to assess the time post-plating at which functional "zippering" of junctions was complete. Co-localization of VE-cadherin, alpha-catenin and beta-catenin to intercellular junctions was shown in a time course to determine the time required after plating to establish strong intercellular adhesion through functional adherens junctions (Fig. 2.8). Immunofluorescence microscopic co-localization of VE-cadherin and associated α– and β-catenin at intercellular junctions indicates formation of functional adherens junctions. High density plating of a single cell suspension of MLEC onto glass coverslips results in closely apposing cells. These cells attach and spread within 1hour.

Cell contact is followed by the functional interaction of proteins on adjacent cells to form adherens junctions. VE-cadherin is upregulated and localization at the cell membrane begins 4 hours after trypsinization and replating (Fig 2.8D). Catenin recruitment is delayed and they can be seen concentrated at junctions by 16 hours (data not shown). The staining pattern of VE-cadherin changes from a jagged sketchy outline of the cell to a tight thin outline of cells, this shift is coincident with the recruitment of catenins. (Vasioukhin et al., 2000). VE-cadherin staining, however, is lost by 21 hours (Fig 2.8.L) I suspect that once tight adhesion is acquired, the epitope is obscured and inaccessible to antibody binding. This indicates to me that a very tight, closely apposed cell junction has formed. Western blotting of confluent endothelial monolayers indicates VE-cadherin protein remains present in the cell, despite the absence of detection by IF (Fig. 2.9, lane 1). Both α- and β-catenin likewise can be detected in these cells by Western blotting (Fig. 2.9). VE-cadherin is significantly reduced by treatment of cells for 4 hours with TNF-α and reduced further by the addition of WEHI cells, a myelomonocyte (macrophage-like) cell line, and a slight reduction in catenins is detected (lanes 3 and 4 respectively) as expected (Del Maschio et al., 1996). The reduction produced by the immune cells was not seen in the absence of TNF-α (Fig. 2.9, lane 2) suggesting that changes in VE-cadherin levels is a consequence of the upregulation and binding of TNF-α inducible receptors.

Figure 2.7: Immunofluorescent Staining for Endothelial Junctional Proteins

Mouse lung endothelial cells were stained according to protocol 6.3 described in the Methods chapter for a panel of common endothelial antigens found in intercellular junctions.

A-D. Confluent passage 4 or 5 cells are stained for proteins associated with cell junctions. β-catenin (A) and α- catenin (B) are intracellular proteins associated at cell junctions with VE-cadherin (C (high power) and D (low power)).

A. β catenin

B. α catenin

C. VE- cadherin (high power)

D. VE-cadherin (low power)

Figure 2.8: Immunofluorescent Detection of Intracellular Junction Assembly

MLEC were plated at a high cellular density (cell number equivalent to a confluent culture ($1x10^3$ cells/mm^2)) and fixed at the indicated time after plating, rinsed and stored in PBS until all samples were collected. All samples were processed identically and simultaneously beginning with the 10% NGS blocking according to the methods for immunofluorescent detection of antigen. VE-cadherin was detected with antibody (Pharmingen)

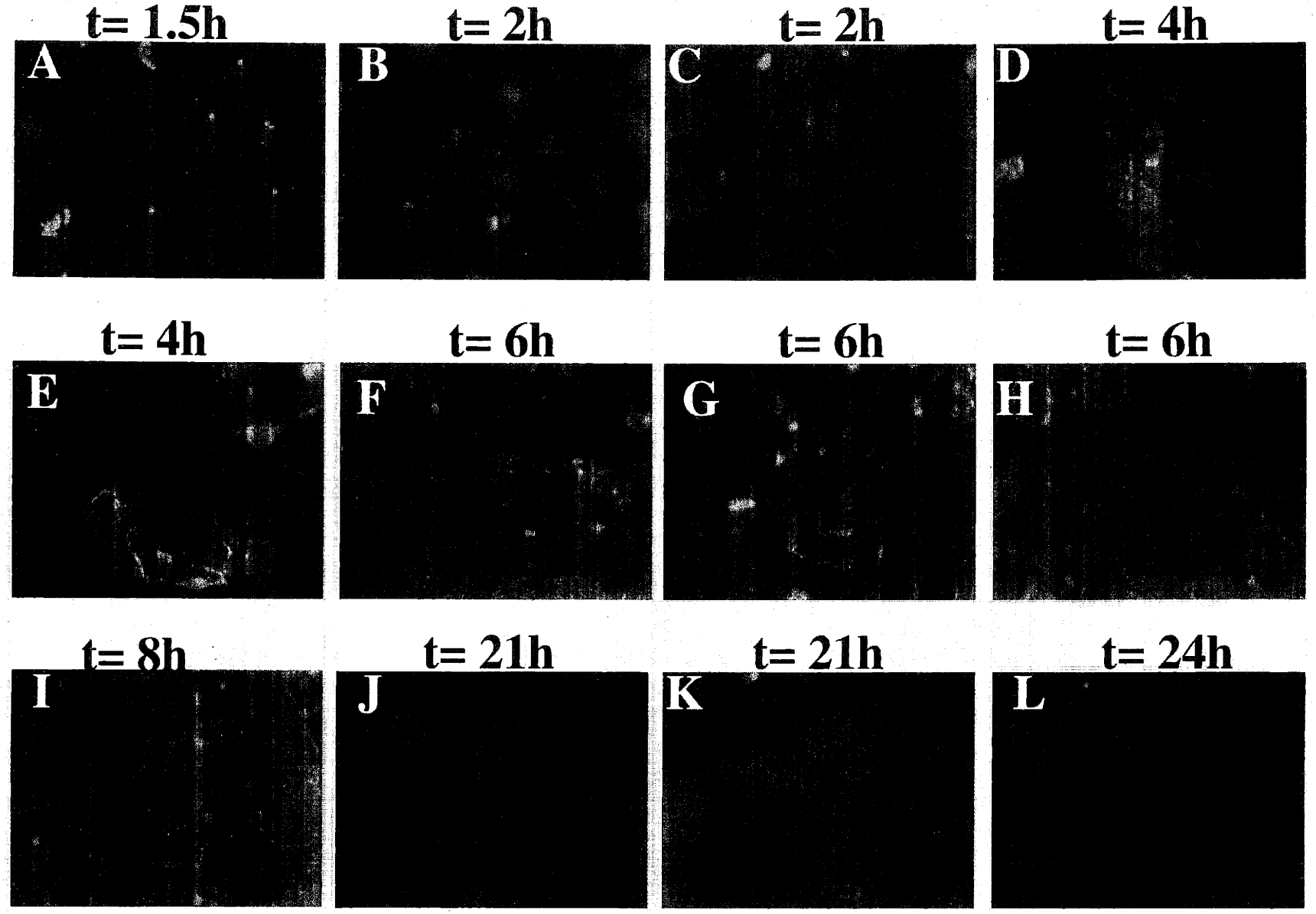
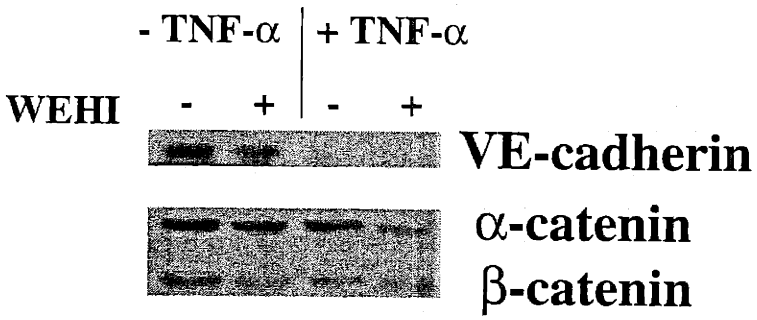

t= 1.5h
A
t= 2h
B
t= 2h
C
t= 4h
D
t= 4h
E
t= 6h
F
t= 6h
G
t= 6h
H
t= 8h
I
t= 21h
J
t= 21h
K
t= 24h
L

Figure 2.9: Western Blotting for Adherens Junction Proteins

Passage 3 wild-type MLEC were plated on 0.1% gelatin coated dishes for 24 hours. Samples were treated with TNF-α for 4 hours (lanes 3 and 4) or fed fresh medium (lanes 1 and 2). WEHI cells (myelomonocytic cell line) were added to MLEC (lanes 2 and 4). Lysates were prepared as described in Methods 6.15. Nitrocellulose membrane was cut horizontally based on a molecular weight standard plot and identical samples were then probed for VE-cadherin as well as catenins.



Integrins, as discussed in the introduction, have widespread expression patterns and are not specific markers for endothelium but are important molecules for the purposes of our studies to characterize. At least 7 heterodimers are expressed on endothelial cells, $\alpha1\beta1$, $\alpha2\beta1$ (Zutter and Santoro, 1990), $\alpha3\beta1$ (de Melker et al., 1997), $\alpha5\beta1$, $\alpha6\beta1$, $\alpha v\beta3$ and $\alpha v\beta5$ and possibly $\alpha4\beta7$ (Brezinschek et al., 1996). However, cell morphology can be used in addition to expression patterns of such integrins as $\alpha v\beta3$ and $\alpha v\beta5$ to determine cell type. Collaborators have assessed the integrin profile of MLEC by immunoprecipitation of surface iodinated cells and shown expression of all integrins tested, $\alpha1$, $\alpha2$, $\alpha5$, $\beta1$, $\beta3$ and $\alpha v\beta5$ (Reynolds et al., 2002). In general, endothelial cells express the $\alpha5\beta1$ and $\alpha v\beta3$ integrins at higher levels than other members of the integrin family. Immunoflurorescent staining localizes integrins $\alpha v\beta3$ and $\alpha5\beta1$ into focal structures, discussed in the thesis introduction (Fig. 2.10). To validate the endothelial nature of the sample, cells were double labeled for Platelet Endothelial Cell Adhesion Molecule (PECAM-1) and $\alpha v\beta3$ (Fig. 2.10 A). $\alpha v\beta3$ in this sample is expressed at a higher level in the "free" edges of the endothelial cell cluster (indicated by arrow in Fig 2.10 A). $\alpha v\beta3$ was localized to the ends of phalloidin-stained F-actin stress fibers in punctate structures, most likely focal contacts (Fig 2.10 B).

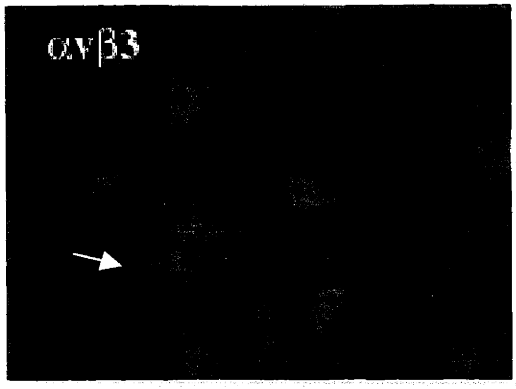
Figure 2.10: Immunofluorescent Localization of β3 Integrin in wild-type MLEC

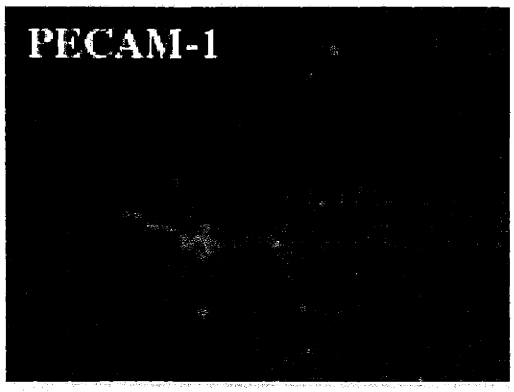
A. Wild-type MLEC fixed 16 h after plating and double stained for αvβ3 integrin (green) and PECAM-1 (red).

B. Wild-type MLEC fixed 21 h after plating and double stained for αvβ3 integrin (green) and actin (red).

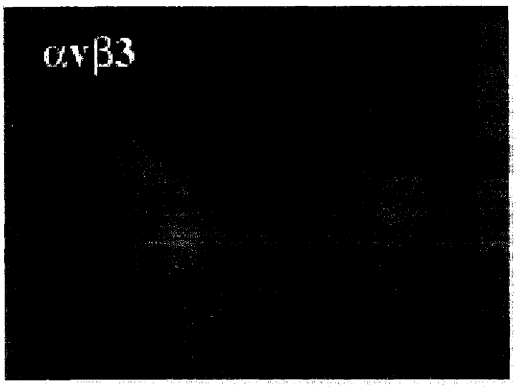
C. Wild-type MLEC fixed 24 h after plating and stained for αvβ3 integrin expression (green) or α5β1 (red)

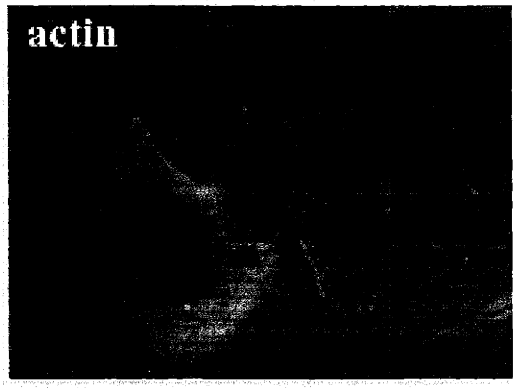
A



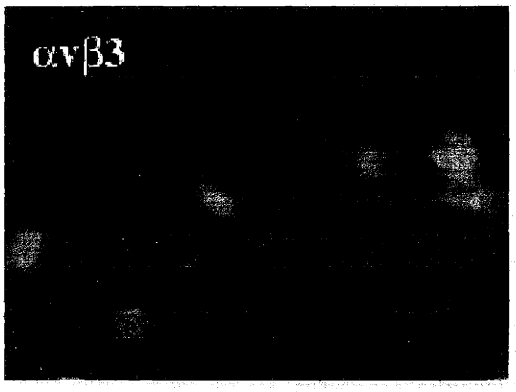


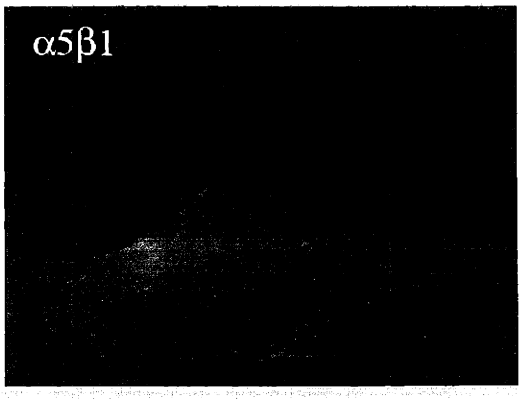
B





C





Validation of Functional Endothelial Cell Population

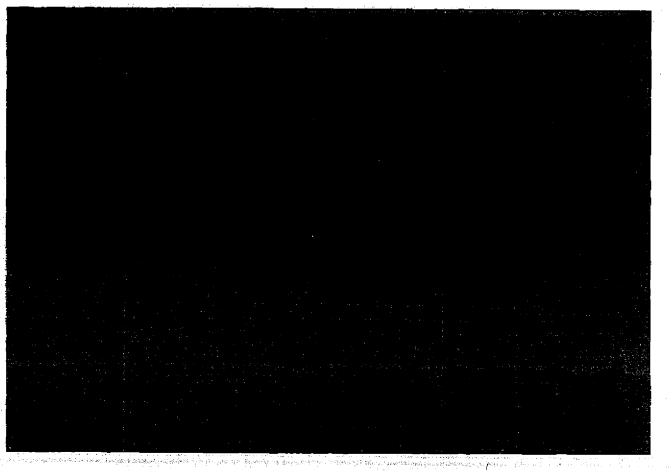
Purified endothelial cells were tested for their ability to form networks of "capillary like" structures. As discussed in the introduction there are many assays in which tube formation can be tested. In all assays tested, MLEC were able to form sprouts and/or networks. Both wild-type and beta 3 -/- MLEC plated at high density on tissue culture plastic form spontaneous networks (Fig 2.11 A and B). MLEC coated onto microspheres in suspension and then embedded in solidified vitrogen (Method 6.5b). After several days in culture endothelial cells began to sprout and form structures resembling tubes or capillaries (Fig 2.11 C). Endothelial cells were also plated at high density on, or sandwiched between, various gelled matrices including collagen, fibrin and matrigel and were able to form tube-like networks. (Fig 2.11 D). These assays indicate that the cultured cells I have isolated maintain the ability to behave as endothelial cells and organize into "capillary-like" structures, a standard assay for angiogenic potential. These data extend the potential utility of harvesting primary murine endothelial cells as I've described.

Figure 2.11: In vitro Angiogenesis Tube Formation Assays with MLEC

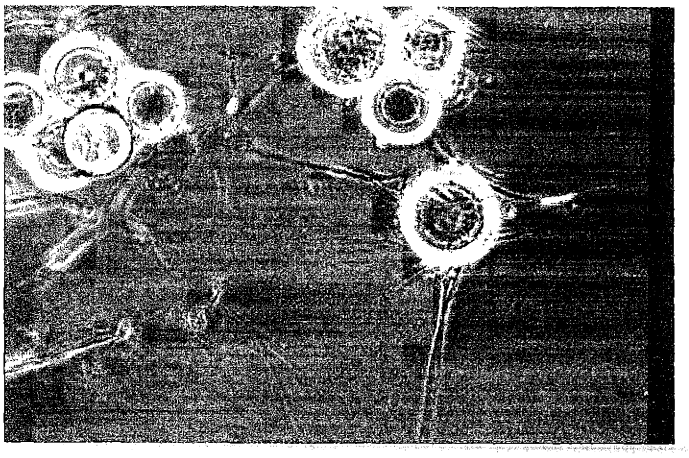
MLEC were tested in several *in vitro* tube-forming assays for their ability to reorganize into network structures. Both wild-type (A) and beta-3 deficient (B) MLEC spontaneously form networks when plated at high density on tissue-culture plastic (Method 6.5a). MLEC plated on microcarrier beads (C) extend processes ressembling vascular tubes when imbedded into clotted fibrinogen (Method 6.5b). MLEC plated on a thick gel layer of 80% vitrogen (D) show vascular-like networks when analyzed by differential microscopy.

A. wild-type MLEC

B. beta 3-/- MLEC



C.



D.

Generation of ts SV40 Lg T expressing MLEC

Animals expressing a temperature sensitive simian virus 40 early-region large T antigen (Jat et al., 1991) were bred into the genetic background of several transgenic lines (Immorto-Mice). The animals harbored a thermolabile large T antigen tsA58 SV40 LgT hybrid construct developed by Jat and Sharp (Jat and Sharp, 1989). Genotyping by PCR confirmed expression of at least one copy of the H–2K b–tsA58 transgene and the appropriate genetic background. Cells were harvested and purified identically to wild-type MLEC. After isolation of a highly purified cell population, cells expressing the LgT Antigen were routinely cultured at 33°C, the permissive temperature, to increase the proliferative capacity of the endothelial population. Cells were shifted to 39°C, the restrictive temperature, to inactivate the LgT transcription factor and induce a differentiated phenotype. Immorto-cells cultured at 37°C maintained a normal endothelial morphology (Fig 2.12) for up to 12 passages, double the proliferative potential of wild-type MLEC cultured at 37°C, before changes in morphology occurred. They had low levels of SV40 LgT in the active conformation as assessed by a conformation-specific antibody and expressed all endothelial markers tested (SV40 staining Fig. 2.13). Cells cultured at 33°C had a slightly altered morphological appearance, no longer maintaining a cobblestone phenotype but a more elongated cell shape that can be seen in certain endothelial cell lines (ie. BAEC). Cells were passaged at 33° for up to 16 passages and retained the ability to shift their phenotype upon shift to restrictive temperature. Cultures were not tested past this passage number. Cells were shifted between permissive temperature, restrictive temperature and 37°C and acquired

the phenotype characteristic of growth at the new temperature within 24 hours. If an event stressed cells at 37°C and they stopped proliferating or showed a decreased doubling time, they were shifted to 33°C in the presence of IFN-γ to increase SV40 LgT expression and stimulate proliferation and expansion of the cell population, which aided in their recovery to a more typical appearance.

Figure 2.12: Morphological Comparison of "Immorto" MLEC

Passage 4 mouse lung cells containing SV40 LgT antigen cultured at 37°C. Minor variations exist in individual preparations and do not indicate generalized differences.

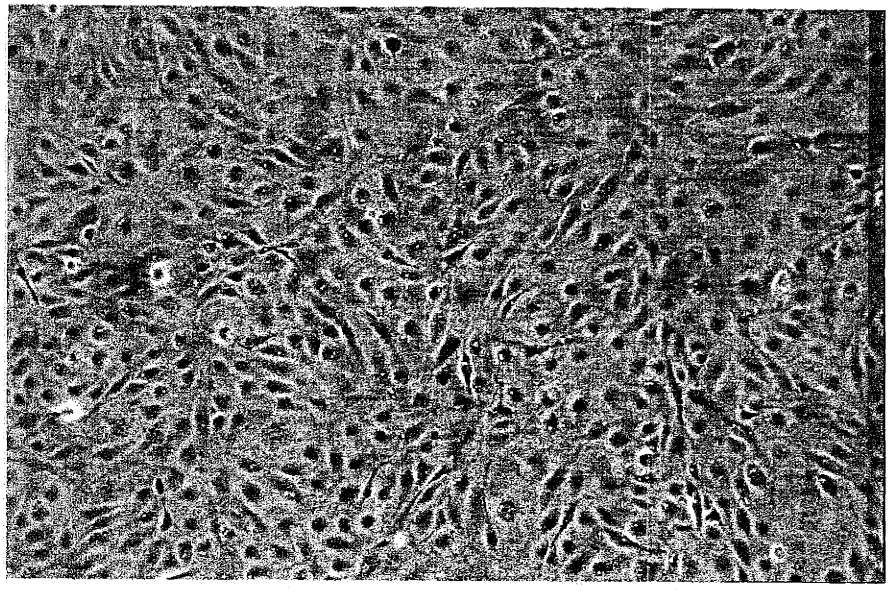
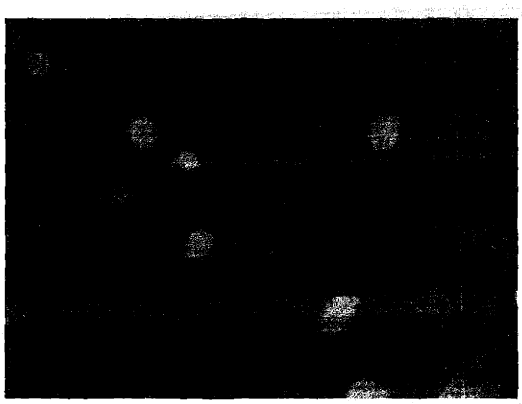


Figure 2.13: Immunolocalization of SV40 LgT Antigen in Immorto MLEC

Passage 4-5 Im+ MLEC were cultured at various temperatures and assayed for the presence of active SV40 LgTAg, using a conformation-specific antibody for immunofluorescence detection. A. Im+ MLEC cultured at 37°C have very little SV40 LgTAg expression. B. Expression of SV40 LgTAg and concentration in the nucleus is variable at 33°C in the absence of IFN-γ. C. Addition of IFNγ induces strong expression and translocation into the nucleus of SV40 LgTAg at 33°C.

A

B.



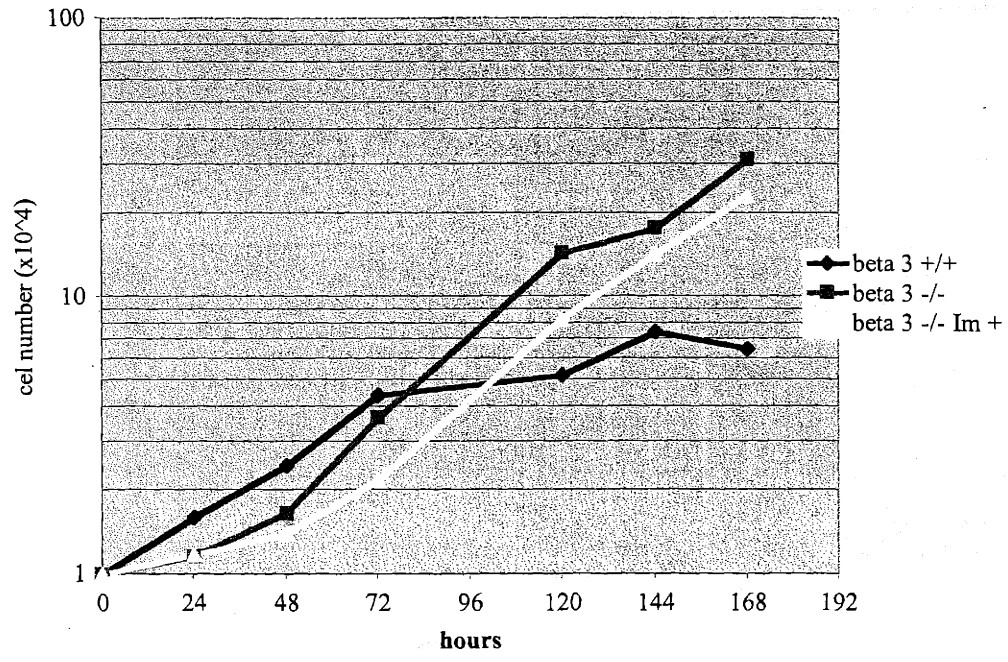
C.

Endothelial cell growth comparisons were made between β3 -/-MLEC or β3 -/- Im^+ (expressing LgT antigen) MLEC and β3 +/+ Im^- MLEC. Immorto cells were cultured at restrictive (39°), permissive (33°) and intermediate (37°) temperatures. The differences between β3 -/- and wild-type cells may indicate slight changes in cell growth due to the absence of beta 3 integrin although minor differences can exist between various MLEC preparations. Beta 3-/- Im + cells cultured at 39° C which induces a cellular differentiation have a slightly slower growth rate compared with wild type non-immorto or β3 -/- Im+ endothelial cells at 37°(Fig. 2.14). Contributing to a decrease in overall population number, cells cultured at 39°C for an extended time appear to lose adhesion more quickly than they divide and repopulate. This trait can also be seen in some preparations of non-immorto beta 3 integrin-deficient endothelial cells grown at 37°C as well. Trypsinization and replating of adherent cells to freshly coated tissue-culture plastic returns their growth to normal. Wild-type cells were less confluent prior to trypsinsization and plating for growth determination, and consequently do not lag after plating, accounting for the slightly higher number of cells harvested at days 1-3. Overall, beta 3-deficient cells at 37°C are slightly more proliferative than wild-type cells, consistent with data that will be shown in chapter 3.

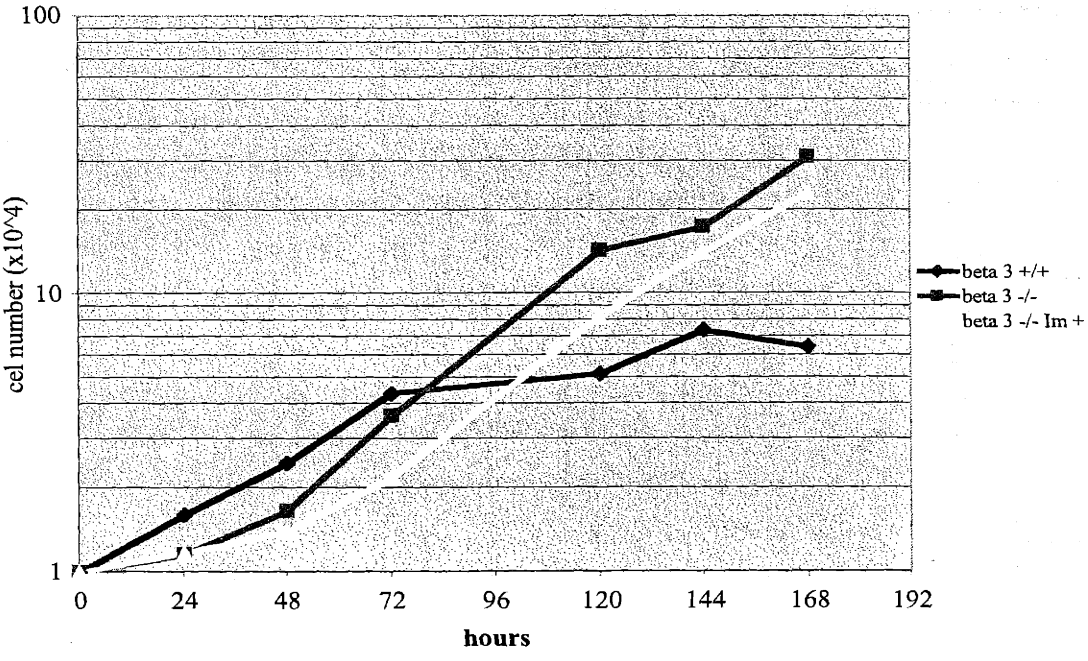
Figure 2.14: Growth Comparison of MLEC

Immorto-positive beta3 integrin-deficient MLEC cultured at 33°, 37° and 39°C are compared with SV40LgT negative wild-type and beta 3 integrin-deficient MLEC cultured at 37°C. Cell suspensions were plated at 1 x 10^4 cells/well in replicate wells of a 6 well dish, trypsinsized at the indicated time after plating and counted using a hemocytometer. The decrease seen at 39° at 168 h in beta3-/- SV40LgT+ cells can be typical of long-term culture of beta 3-deficient MLEC without passage onto newly coated dishes. Cells will continue to expand when replated. Comparison of cells cultured at 37° (B) shows that beta 3-null MLEC growth is enhanced regardless of the presence of SV40LgT.

A.


MLEC Growth at 37°
100
10
1
cel number (x10^4)
0
24
48
72
96
120
144
168
192
hours
beta 3 +/+
beta 3 -/-
beta 3 -/- Im +

B.


MLEC Growth at 37°
100
10
1
cel number (x10^4)
0
24
48
72
96
120
144
168
192
hours
beta 3 +/+
beta 3 -/-
beta 3 -/- Im +

Section 2.3: Discussion

I have described in the preceding pages a reproducible method to obtain a nearly pure population of endothelial cells judged by characteristic morphology, protein expression and angiogenic function. The populations of cells harvested were consistently greater than 97% pure endothelial cells and, as in the case of the examples presented in this chapter, often greater than 99% endothelial cells based on FACS analysis of a panel of cell-specific antigens. As mentioned earlier, despite the presence of several antigens on other hematopoietic lineages, the combination of marker expression and cell morphology, was sufficient to confidently declare this a homogeneous population.

Several publications report the isolation of primary human (Hewett and Murray, 1996; Springhorn et al., 1995) or rat (Demeule et al., 2001; Jones et al., 2000; Matsubara et al., 2000) endothelial cell lines employing PECAM-1 coated magnetic bead purification and several labs have attempted this method on murine tissues with variable outcomes ((Dong et al., 1997), pers. comm.) Bittinger (1996) has reported low-level constitutive expression of PECAM-1 on mesothelium, a cell type which could possibly contaminate endothelial population from the lung. In addition, I have found decreased PECAM-1 expression on the cell surface following certain protease treatments which may influence yield where PECAM selection of enzymatically digested tissues is attempted.

Primary cell lines are beneficial to research yet troublesome to work with. They present unique complications in experimental design and can be difficult to characterize. They are inherently as closely related to the in vivo source from which they originate as possible and do not have introduced mutations that can be found in many established or transformed cell lines. Since they originate from a given and usually small number of individuals, they can be highly variable. It is important to determine which steps in the isolation can generate variability and minimize this contribution to the final cell population. I have found in isolating MLEC that the number of positive selections and the inclusion of a negative selection, although important, can vary greatly without compromising the final purity. The choice to include both mechanical as well as chemical dissociation techniques was made based on the morphology of the lung. Due to the very high number of microcapillary beds which are sensitive to pressure and the difficulty to contain the circulation by clamping, the vascular component can not be easily perfused with proteolytic enzyme as is done in isolation of human umbilical vein endothelial cells. The selection of collagenase was based on literature of other endothelial purification methods as well as knowledge that collagen is a major constituent of vascular basement membrane. Other enzymes with less specificity, ie trypsin, dispase, or proteinase K, can be considered but will likely cause additional stress on the endothelial cells.

General cell culture principles are important to consider once a single primary cell suspension is achieved. Any cell preparation can benefit by plating onto an appropriate substrate thus improving yield. Gelatin coating of tissue-culture plastic will enhance cell adhesion of endothelial cells but sometimes additional substrate coating is necessary,

particularly if cultured cells are deficient in specific adhesion receptors. In the case of beta 3- or beta 3/beta 5-deficient endothelial cells, fibronectin and a proprietary solution, Vitrogen, (mostly Type I Collagen), was added to the gelatin coating. Importantly, while culturing any cell type, optimal feeding schedules and proper oxygenation are required. Certain cells produce autocrine factors which are beneficial to cell growth. In these cases, it may be beneficial to use "conditioned" media when feeding cells; conditioned media is that which has been incubated on cells and contains useful secreted factors. Conditioned media mixed in an optimal ratio with fresh media in these cases provides a complete nutritionally balanced media for cell growth. MLEC cultures were treated with conditioned media and showed no growth enhancement, although further testing could be done.

As expected by design, all cells in the final population expressed ICAM-2 and consistently express high levels of PECAM-1. As reported in the results section, not all cells with endothelial morphology express ICAM-2 at initial passages. This is in apparent conflict with reports of constitutive expression patterns but may in fact be more consistent with reports of down-regulation of ICAM-2 in activated endothelium (Langley et al., 1999; McLaughlin et al., 1998). As mentioned in the results and discussed further below, the purification method outlined in this chapter induces the gene expression of ICAM-1, an effect seen in activated endothelium, and may likewise shift some cells to express ICAM-2 at a level seen in activated endothelium. Although the mechanism has not been elucidated, circumstantially, these related family members are regulated in opposite fashion and this is somewhat consistent with what I have seen. ICAM-2

expression does appear to be activated in subsequent passages whereas no decrease in ICAM-1 expression has been noted in later passages.

Variations in expression of characteristic proteins such as vWF and ICAM-1 on cells purified by this method should be considered if these molecules are suspected to be critical in future experimental design. Their altered expression does not compromise the validity of this method of isolating primary cells but must be recognized if these molecules may impact a particular study. The absence of ubiquitous or sustained vWF expression in culture is not exceptional and is a phenomenon common to many cultured endothelial cells (e.g., Dorovini-Zis and Huynh, 1992; Guan et al., 1999; Gumkowski et al., 1987; Page et al., 1992)}. This absence is paralleled by the absence of constitutive P-selectin expression (immunofluoresence data not shown) suggesting the complete absence of Weibel-Palade bodies, secretory granules in which both molecules are stored, consistent with data suggesting that multimeric vWF directs the generation of these organelles and anchoring therein of P-selectin (Hop et al., 2000). Unexpected constitutive expression of ICAM-1 was not a point of concern for my studies and is an attribute seen in other primary endothelial cell lines (Hauser et al., 1993; Page et al., 1992; Silverman et al., 2001) but this means that the cultured MLEC should be viewed as partially activated.

These changes may be indicative of an activated phenotype or at least a modified expression profile. These changes do not discount the importance or utility of these cells. It is possible to attempt to modify the protocol to limit these alterations in cell activation. Limiting the manipulation of cells generally limits the activation of them. Chen et al. (1995) have described a method for non-mechanical, non-enzymatic isolation of

endothelial cells from outgrowths of lung explants. This method purports to cause no cellular activation but yield is much lower than by this method. Magnetic bead sorting consistently yields higher endothelial cell numbers per animal than other methods

Although not supported by literature searches, the changes in ability to detect VE-cadherin in MLEC junctions were reproducible. The sustained expression and localization of alpha and beta catenin at intercellular junctions imply functional cadherin engagement. This could be VE-cadherin but it would be formally possible that another cadherin, N-cadherin, is upregulated. Western blots indicate that VE-cadherin protein can still be found in cell lysates and makes this possibility less likely. This time-course analysis apparently establishes that MLEC can form a functional adherens junctions in less than 24 hours and cellular permeability could be tested in several ways including electrical resistance to confirm this.

In addition to wild-type and beta 3-deficient endothelial cells used in my studies in this thesis, I was able to successfully harvest several cell lines of different genotypes. Due to the particular interest in the similarities of αv integrin partners beta 3 and beta 5 (which will be discussed in detail in the following chapter), isolation of doubly deficient endothelial cells seemed valuable. Since the majority of αv-null animals are embryonic lethal and the two major αv partners are beta3 and beta 5 it was highly surprising that animals generated by crossing beta 3 null mice and beta 5 null mice had no gross developmental defects. These doubly deficient cells have been used in shear stress experiments by my collaborator, T. Nagel, at Genentech. Upon observing little change in shear stress response in beta 3-deficient endothelial cells, beta 3/beta 5-deficient cells were tested to determine if either may play a role in mechanotransduction. These cells

potentially will be used in additional in vitro angiogenesis assays as will be discussed in the next chapter.

Murine endothelial cells can be valuable tools in elucidating the role of many cellular proteins. I have helped others in the lab (by advising and occasionally manually) to use this technique to successfully isolate cells from animals deficient for FN (personal communication, S. Burrows), alpha 5 integrin (personal communication, K. Goh), TNFR/relA (personal communication, E Alcamo) as well as isolating cells from tie-2 GFP transgenic line (Motoike et al., 2000) expressing green fluorescent protein in an endothelial-specific fashion myself. Chimeric animals have been generated by blastocyst injection of FN null or alpha 5 null embryonic stem cells (Taverna et al., 1998). Both deficiencies result in embryonic lethality and both adhesion molecules are very important for proper vascular development, as discussed in the thesis introduction. Purification of a mixed genotypic population of endothelial cells followed by antibiotic selection yielded a population of null endothelial cells. Although the studies on these cells are not complete, preliminary evidence suggests that cells lacking alpha 5 integrin do not organize FN matrix and form tubes less efficiently in spontaneous tube forming assays (similar to Method 6.5a) and FN null endothelial cells are being tested for their ability to associate with wild-type or null dermal fibroblasts in a 3- dimensional co-culture assay.

The ability to isolate endothelial cells expressing GFP allows one to think of the possibilities of using labeled endothelial cells in any number of biological assays. These cells express green fluorescent protein under the endothelial-specific tie-2 promotor expressed constitutively in endothelium (Constien et al., 2001). Although purified by magnetic bead sorting as outlined in this chapter, the expression of GFP raises the

possibility of sorting quickly by FACS. High-speed cell sorters are now available (Mo Flo, Cytomation, Fort Collins, CO) but have not been tested in purification of MLEC. These instruments can analyze and sort 30,000-40,000 cells/sec nearly ten times faster than previous equipment. This advance may eliminate the disadvantages of FACS purification of sensitive endothelial cells. The duration of the FACS procedure and the length of time between removal of cells from the animal and cell adhesion onto a culture substrate was postulated as the stress which adversely impacts recovery of endothelial cells. Analysis with newer instrumentation could certainly distinguish if this hypothesis was in fact correct or if high shear involved in sorting may be to blame. If high speed FACS sorting proves to be a viable method of purification, Tie-2 GFP mice and the ability to cross this trait into other genetically modified animals could greatly enhance endothelial cell preparation.

Beyond our laboratory, I have made this protocol available and several other labs have used this method to purify various transgenic cell lines. cd39/ATP diphospho-hydrolase, an enzyme which hydrolyzes beta and gamma phosphates of tri- and di-phosphonucleosides to generate nucleoside monophosphates, can be found on human endothelial cells, platelets and selected monocyte, NK, and megakaryocyte cell lines. Purinergic signaling may play a role in hemostasis, thrombosis, inflammation and apoptosis and the activity of CD39 may therefore play a role here(Koziak et al., 1999). Knock-outs were generated and endothelial cells were purified and analyzed (personal communication and Enjyoji et al., 1999). Cells have also been made from IAP -/- mice and the investigator was able to clonally select cells which were maintained in culture for up to 20 passages. (M. Johansen, Brown Lab). Integrin-associated protein (IAP) was

isolated from placenta based on its affinity for αvβ3. It is implicated in communication between cell surface receptors and is important for cross talk. Null cells provide a system in which one can ask if IAP is absolutely necessary for this function. The Kalluri lab at Beth Israel-Deaconess Medical Center is attempting to understand the roles of antiangiogenic factors through analysis of knockouts in associated molecules, for instance MMP-2 and IFN-α. This collection of transgenic lines from which MLEC have been isolated demonstrates the wide utility of this protocol. I have yet to find an example of a viable animal from which endothelial cells cannot be harvested in this fashion.

Chapter 3: Effects of Beta 3 Integrin Deficiency on VEGF Responsiveness

Section 3.1: Introduction

The need for the development of a sufficient blood supply to tumors involves a complex set of factors which may depend critically on the production and action of VEGF. VEGF is clearly upregulated in many tumors, and for many reasons. As tumors grow, the distance between the center of the tumor and host vessels becomes so great that hypoxia results (see Chapter 1: Introduction). Multiple pro-angiogenic cytokines, including vascular endothelial growth factor (VEGF) and the fibroblast growth factors, are induced by this hypoxia as well as by macrophage infiltration in the tumor microenvironment (Eatock et al., 2000). Evidence has suggested that, in fact, growth factors will induce expression of each other to enhance the pro-angiogenic stimulus in proliferating tumors (EGF induces VEGF Maity et al., 2000; Ravindranath et al., 2001). The increase in vascular permeability in tumors concomitant with the induction of endothelial cell proliferation is attributed to the induction of VEGF/VPF, even in experimental conditions where VEGF has not been provided as the pro-angiogenic stimulus (Bergers et al., 2000). In support of this, a number of putative pro-angiogenic factors including small molecules (e.g. prostaglandins, adenosine) as well as additional cytokines (e.g. TGF-alpha, bFGF, TGF-beta, TNF-alpha, KGF, PDGF) have been shown to upregulate VPF/VEGF expression (reviewed in Brown et al., 1997). In addition to stimulating the proliferation of vascular endothelial cells, these growth factors stimulate the synthesis of proteases such as urokinase-type plasminogen activator (uPA) and the

matrix metalloproteases (MMPs), which presumably result in digestion of the extracellular matrix and allow endothelial cell invasion and thus drive the progression to angiogenic tumors (Cross and Claesson-Welsh, 2001; Eatock et al., 2000).

The regulation of VEGF/VPF expression during tumor progression may depend on additional diverse proteins expressed from activated oncogenes, mutant or deleted tumor suppressor genes, cytokine activation, hormonal modulation (Claffey and Robinson, 1996). Friedlander presented data which he proposed supported a model in which beta 5 integrin signaling is necessary for VEGF-mediated signaling, which will be discussed further below. Relevant to this thesis, we have generated recent evidence that indicates alpha v beta 3 integrin can also regulate VEGF expression and potentially, its activity; animals lacking beta 3 integrin have an increase in serum levels of VEGF (determined by ELISA) and an increase in VEGFR-2 on the endothelial cell surface (Reynolds et al., 2002 and Hodivala-Dilke, K. personal communication). This *in vitro* observation was realized after analysis of pathological angiogenesis in beta 3-deficient animals revealed an expansion of vessel number. Comparisons of several tumor cell lines and models support the conclusion that tumors removed from beta 3-deficient animals are larger and have an increase in vessel density, although exceptions have been seen in the RIP-Tag model of spontaneous pancreatic islet tumor formation in beta 3-null animals (K. Rubin, personal communication). Likewise the number of vascular glomeruli in the eye was doubled in beta 3-deficient neonates with wild-type neonatal mice in hypoxia-induced retinal neovascularization (Reynolds et al., 2002).

In addition to β 3, endothelial cells also express β5, another αv partner. These two heterodimers have long been implicated as important regulators of angiogenesis. A

significant body of literature links beta 3 and beta 5 integrins with the morphogenesis of blood vessels. Interestingly beta 5 integrin-deficient animals, like beta 3-null animals, are viable and fertile and have no defects in wound healing or adult angiogenesis (Huang et al., 2000). Even more interesting is the lack of defects in the β3/β5 double knockout mice. These animals are viable and fertile, although they do exhibit increases in pathological angiogenesis as is seen in the β3 single knockout. This phenotype is not close to the severity of the αv subunit knockout, suggesting yet another αv integrin partner is contributing to developmental angiogenesis.

The increase in angiogenesis and upregulation of VEGF and VEGFR-2 in beta 3-deficient animals are possibly the result of a loss of cross-talk from αvβ3 integrin. Cross-talk as well as the integration of signals from multiple cell-surface receptors must be invoked in many cases to explain the complexity of cellular responses. Rarely do cells encounter only a single isolated signal that impacts its processes. It has been reported for some time that integrins and growth factor receptors impinge on each other's ability to signal and effect downstream events; they can both amplify and attenuate signals. The integration of multiple signals from the cell surface allows a comprehensive cellular response to environmental changes to occur.

The regulation between these two receptors can function in either direction. Growth factor signaling can also regulate the transcription of integrins (Harwood et al., 1999; Klein et al., 1996), but this too may be dependent on additional cellular signals to generate specificity of integrin upregulation. Fibroblasts treated with PDGFBB upregulate α3-and α5- integrins in fibrin and fibronectin-rich environments yet α2

integrin is maximally induced in both stressed and relaxed collagen (Xu and Clark, 1996).

Friedlander et al. (1995) have looked at the effects of supposed blocking reagents to αvβ3 and αvβ5 on bFGF- and VEGF-induced angiogenesis in both the rabbit corneal or CAM angiogenesis assays. In the presence of αvβ3 specific reagents, bFGF-induced angiogenesis is inhibited while in the presence of αvβ5 specific reagents VEGF-induced angiogenesis is inhibited. Pro-angiogenic growth factors depend on specific integrin signaling for their effectiveness. They conclude that signaling through αvβ3 must occur for endothelial cells to be responsive to bFGF in rabbit corneal or CAM angiogenesis assays. They surmise that their reagent binds beta 3, no pro-angiogenic signal occurs and thus bFGF-induced angiogenesis is blocked. Likewise their beta 5-specific reagent blocks a necessary signal for VEGF-induced angiogenesis.

Section 3.2: Results

Growth of Primary Mouse Lung Endothelial Cells

MLEC were harvested as described in Chapter 2 from wild-type and beta 3-deficient animals and cultured to assess differences due to the absence of beta 3 integrin. The yields of beta 3-null endothelial cells appeared to be lower than wild-type endothelial cells. Upon further observation, it appeared that beta 3-deficient cells were not attaching as well to gelatin-coated tissue-culture plastic and therefore not surviving. This deficiency in adhesion to gelatin-coated plates was compensated by plating cells instead onto a matrix of fibronectin, Vitrogen®, and gelatin and the yields of beta 3-null MLEC preparations were then comparable to wild-type endothelial cells plated onto the same mixture. To test for a specific matrix adhesion deficiency, cell attachment to various substrates was tested. Beta 3-deficient and wild-type MLEC exhibited comparable adhesion to fibronectin, collagen and laminin. Adhesion to vitronection, however, was minimal in beta 3-null MLEC whereas wild-type MLEC exhibited robust adhesion (Fig. 3.1, assay performed by Reynolds, L. and Hodivala-Dilke, K.).

The growth of beta 3-null and wild-type MLEC was compared. Beta 3-deficient cells cultured at 37°, 5% CO2, in normal medium (see Methods 6.1) grew slightly better than their wild-type counterparts (Fig. 3.2). Due to the complex nature of the medium and unknown composition of the added endothelial mitogen (Biomedical Technologies Inc.), this result was not further analyzed to determine if a particular growth factor was

responsible for this effect. Instead, growth was assessed in angiogenesis assays bearing a closer resemblance to the *in vivo* setting.

Figure 3.1: Adhesion Assays of Beta 3 -/- and Wild-Type MLEC to Various Matrix

Wild-Type (□) and β3 -/- (■) MLEC were plated onto fibronectin (10μg/ml), collagen, laminin-1, vitronectin or control uncoated dishes. Cells were allowed to attach to various substrates for 1 hour. Adherent cells were counted cells after staining with crystal violet (see Method 6.8) (assay performed by Reynolds, L.).

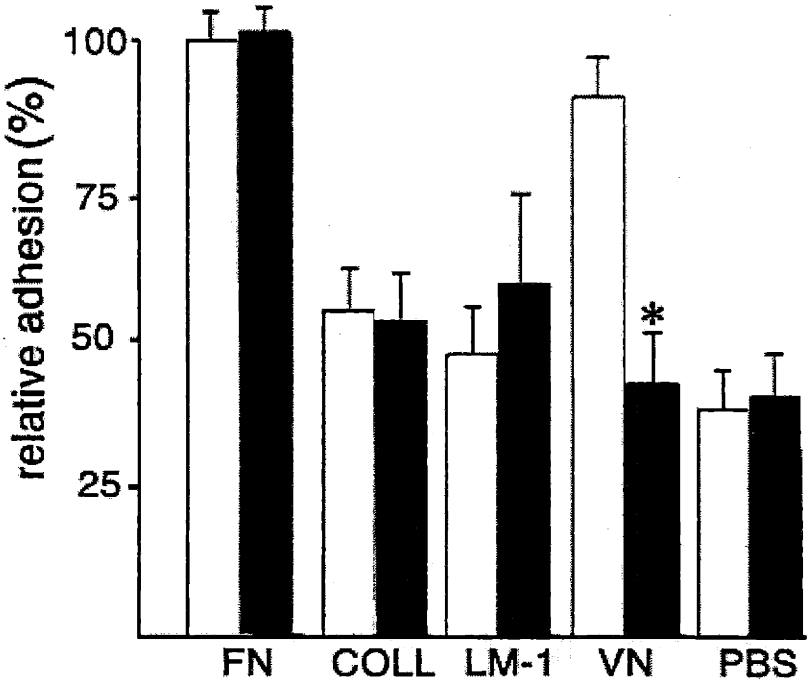
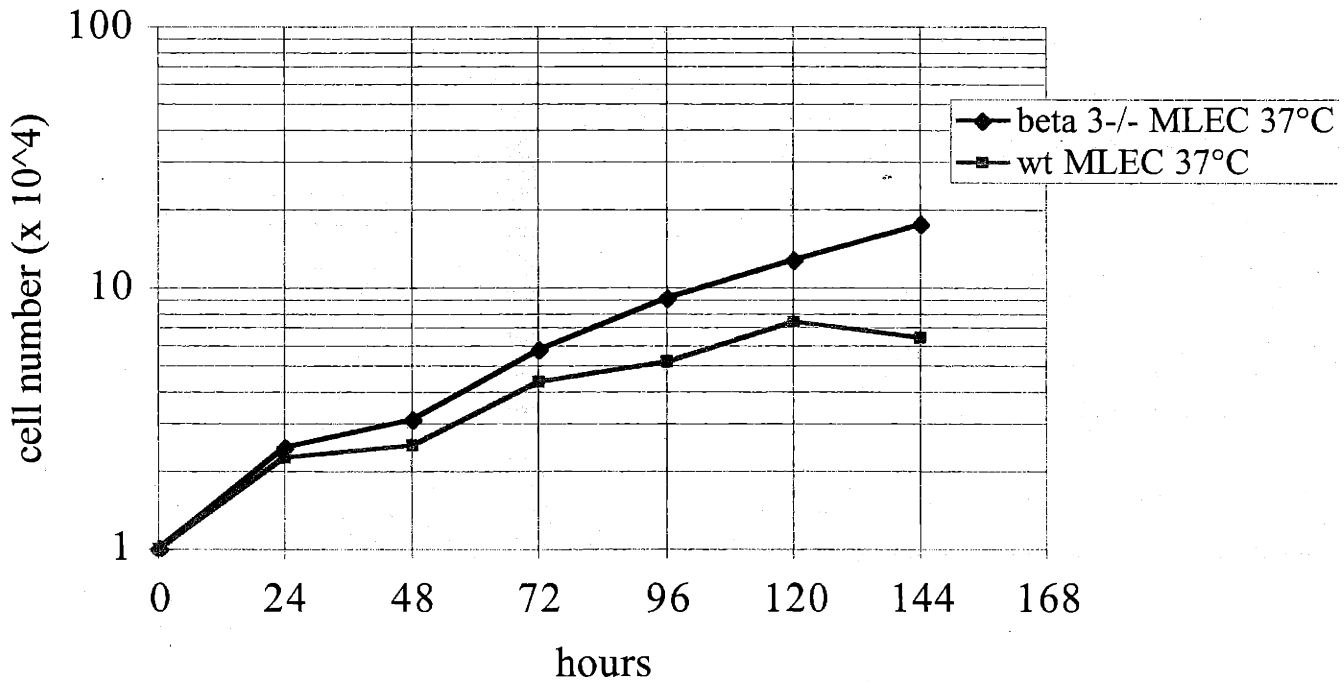


Figure 3.2: Growth Comparison of Beta3 integrin-deficient MLEC and Wild-Type MLEC

Cell suspensions of passage 4 MLEC (wild-type ■ or beta 3-/- ◆) were plated at 1 x 10^4 cells/well in replicate wells of a 6 well dish, trypsinsized at the indicated time after plating and counted using a hemocytometer.



Aortic Ring Growth in Explants from Wild-Type and β3 deficient Mice

Aortae from wild-type and beta 3-deficient animals were dissected as described in the Methods 6.6 and sections were embedded in Matrigel containing growth factors. Three days after explantation sprouts would begin to form in highly responsive environments and sprouts would continue to form throughout the additional 4-10 days of culture. These sprouts would elongate, fuse and branch in a similar fashion to what would be expected from de novo angiogenesis. Surprisingly, there were no differences in sprouting from cut edges or intact aortic walls, sprouting reproducibly originated equally from all edges. Figure 3.3 contains photographs of representative aortic rings in either highly angiogenic or poorly angiogenic environments. The response to VEGF was dose-dependent for both wild-type and β3-null samples. Addition of VEGF, either 30ng/ml or 10ng/ml, to beta 3-null explant cultures induced more extensive vascular sprouting than addition of the same concentration of this growth factor to wild-type explants (Fig 3.4). Addition of 30ng/ml VEGF to wild-type samples induces sprouting comparable to that seen with 10ng/ml VEGF treatment of beta 3 -/- aortic rings. Whereas treatment of beta 3-null explants with this concentration of growth factor induced nearly 3 times more vascular sprouting (18.7 +/- 3 compared with 6.5 +/- 2, $n = 8$, $p < 0.001$).

Culture of aortic explants from both wild-type and beta 3-deficient mice in the presence of varying doses of bFGF stimulated more vascular sprouting than treatment with a range of VEGF doses (10-100ng/ml) (compare Figure 3.3 B and C). Experiments have been inconclusive as to whether there is a dose response to bFGF under 3 tested concentrations (30, 50 and 100ng/ml). Experiments have also been inconclusive as to

whether there is a difference between bFGF-induced sprouting in wild-type and beta 3-deficient aortic explants. Likewise no differences in sprouting were seen between wild-type and beta 3-null explants in medium containing normal endothelial cell culture media (20% fetal bovine serum) (Figure 3.4).

Figure 3.3: Representative Photographs of High and Low Sprouting Aortic Rings.

A. Diagram of section of aorta for orientation purposes. Rings are laid into Matrigel on their side with lumen parallel to the culture dish bottom. When viewed from the side the lumenal area does not appear to collapse. Generally both "upper" and "lower" edges of the cut surface can be independently brought into focus.

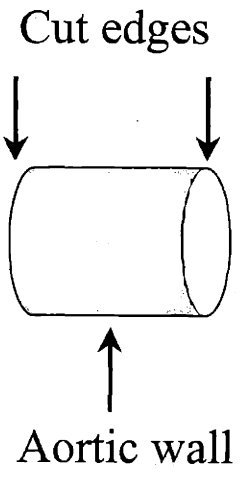
B. Wild-type aortic ring embedded in Matrigel and fed with low serum (2%) and 10ng/mlVEGF after 7 days of culture. A low number of structures are shown sprouting from the aortic walls.

C. Wild-Type aortic ring embedded in Matrigel and fed with low serum (2%) and 100ng/ml bFGF after 7 days of culture. Many vascular sprouts are seen in varying degrees of complexity from the aortic wall.

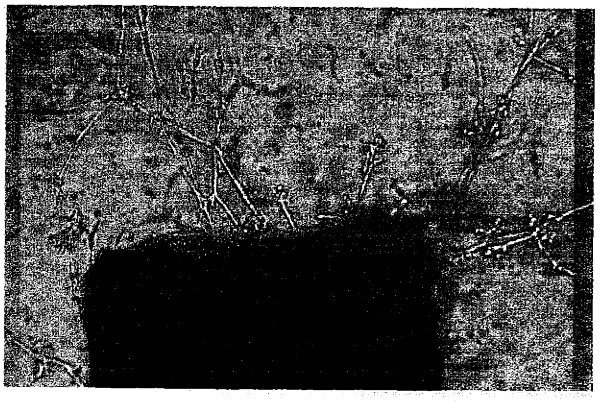
D. Wild-type aortic ring embedded in Matrigel and fed with low serum (2%) and 30ng/ml VEGF after 7 days of culture. Sprouting is comparable to that seen in E.

E. Beta 3 -/- aortic ring embedded in Matrigel and fed with low serum (2%) and 10ng/mlVEGF after 7 days of culture. Many vascular sprouts are seen in varying degrees of complexity.

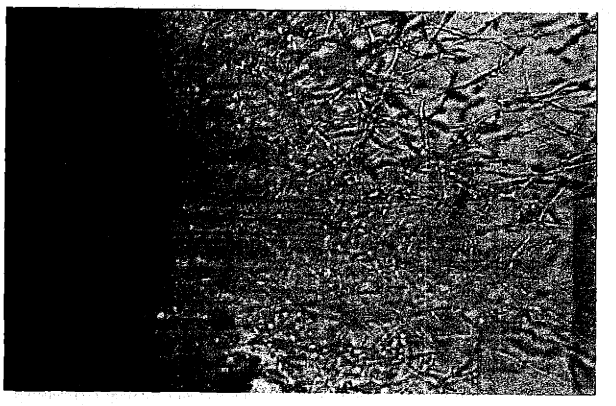
A.



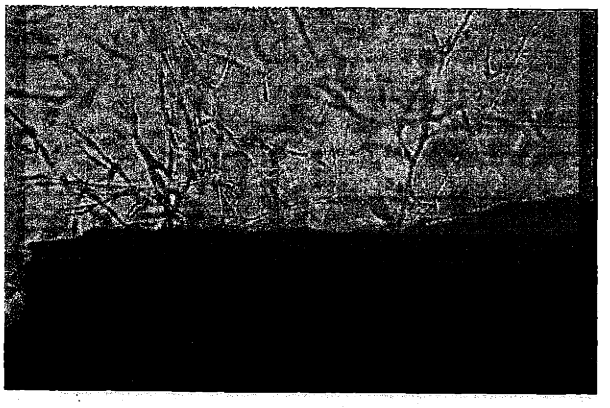
B.



C.



D.



E.

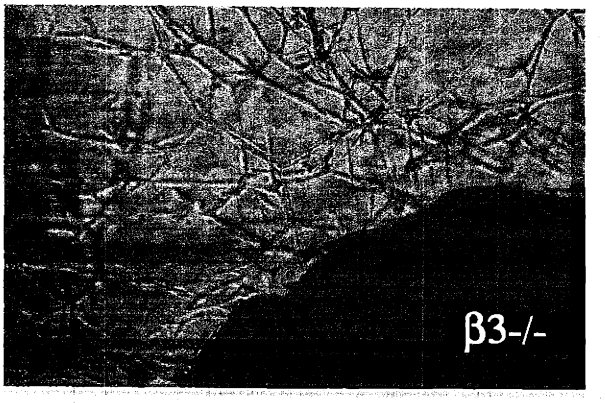
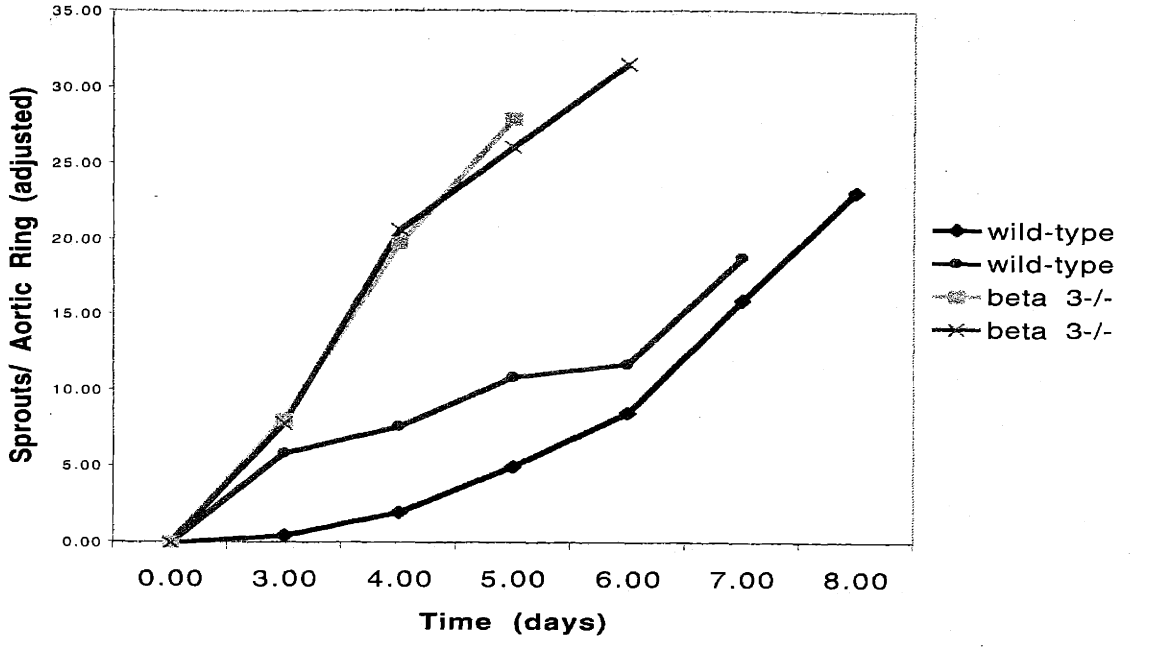


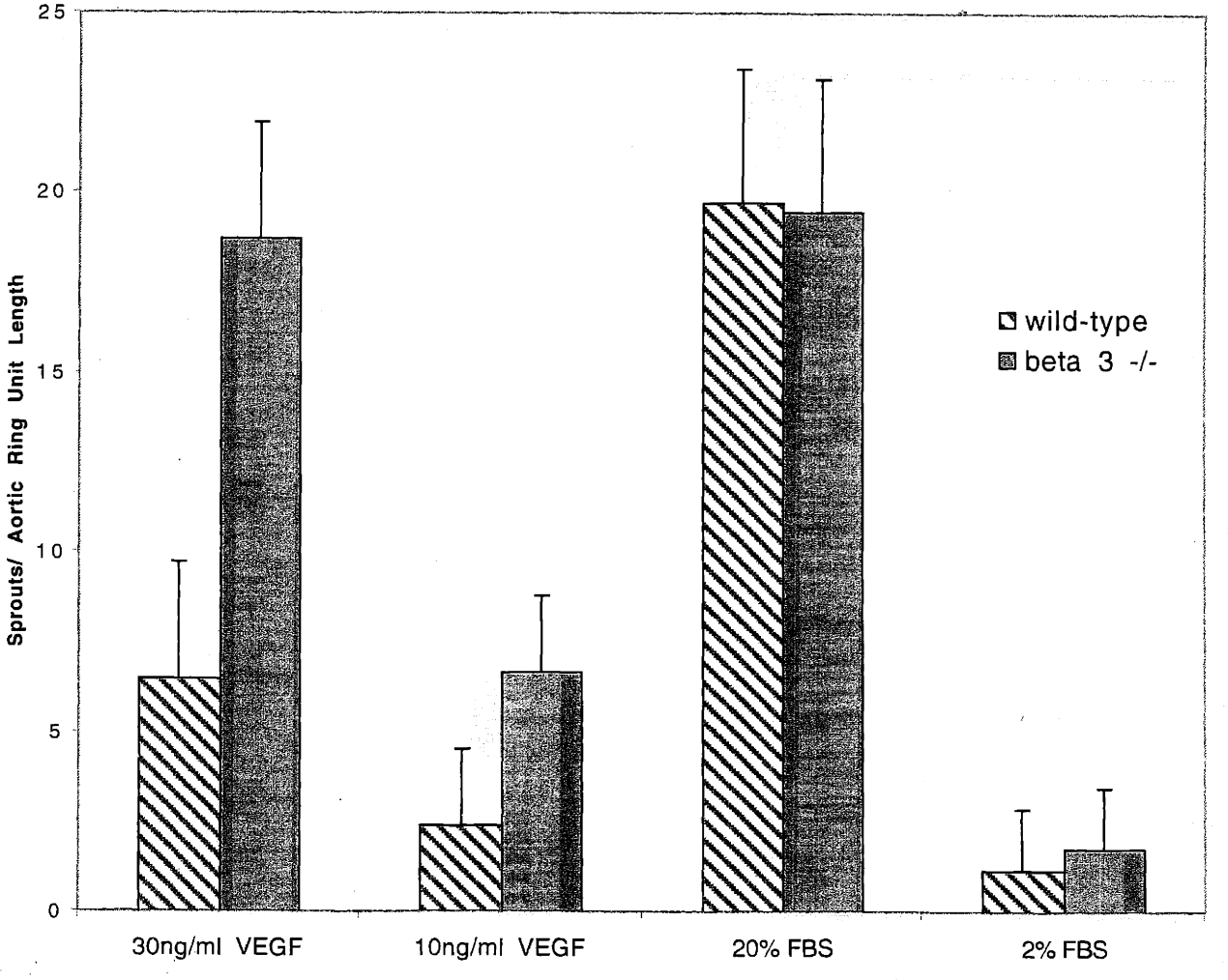
Figure 3.4: Beta 3-deficient Aortic Rings exhibit more VEGF-induced Sprouting

The graph in A. represents a typical time course of sprouting for wild type and β3-/- aorta in the presence of 30ng/ml VEGF. The chart in B represents the total number of sprouts generated per aortic ring before any regression was seen (day 6-8). Rings were incubated with VEGF as indicated below graph. Counts were adjusted for differences in size of the ring as noted in Methods protocol 6.6. Each column represents growth from > 5 rings from > 2 animals per genotype. The differences in growth from control aortic rings and beta 3-deficient aortic rings were statistically significant ($p < 0.001$).

A.


35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Sprouts/ Aortic Ring (adjusted)
0.00
3.00
4.00
5.00
6.00
7.00
8.00
Time (days)
wild-type
wild-type
beta 3-/-
beta 3-/-

B.


25
20
15
10
5
0
Sprouts/ Aortic Ring Unit Length
wild-type
beta 3 -/-
30ng/ml VEGF
10ng/ml VEGF
20% FBS
2% FBS

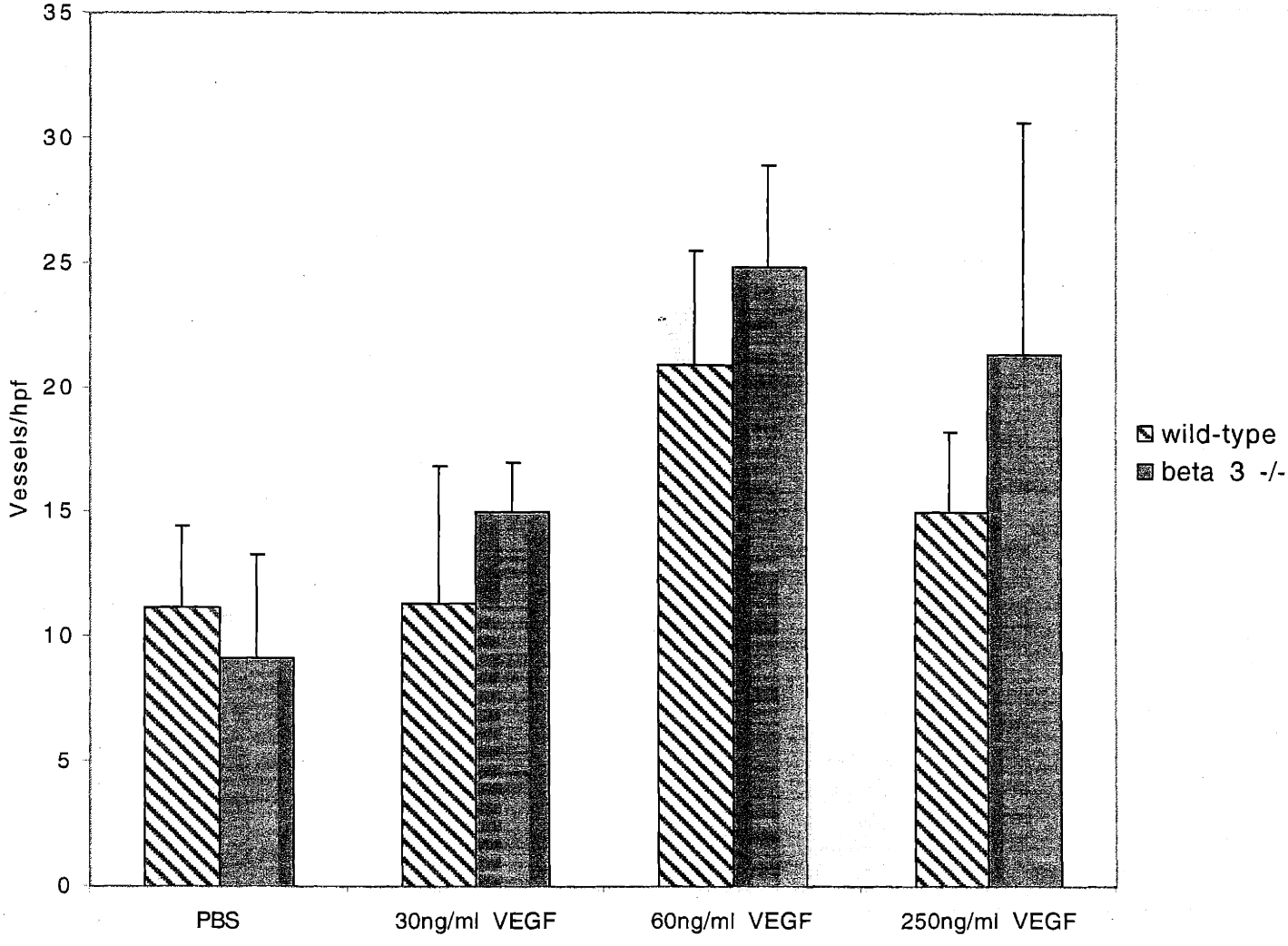
Angiogenesis in Matrigel Plug Inserts in Wild-Type and β3 Deficient Mice

Wild-type and beta 3-deficient animals were injected subcutaneously with a mixture of Matrigel, growth factor and heparin as described in the Methods section, protocol 6.7. Plugs were kept in the animals for 7 days before harvesting, fixing and sectioning. Histological sections of Matrigel were stained with hemotoxylin and eosin (H & E) and vascular structures were counted in a masked fashion, totals graphically represented in Fig. 3.5. Lumen-containing structures, with or without blood cells in the void, were counted as vessels. If a transverse section of a vessel was seen, this too was counted. Single H & E stained cells were not counted unless a lumen could be seen, these are most likely infiltrating leukocytes.

Responses to VEGF were dose-dependent and, as seen in the aortic ring assay, at all doses beta 3-deficient animals promoted a more vigorous response. Heparin was also able to vary the induction of vascularization of the Matrigel plug as reported. To ensure the effects that were seen were due to the VEGF concentration, the heparin concentration was kept at 20U/ml. The differences seen at 30ng/ml VEGF, 20U/ml heparin, although subtle are statistically significant ($p < 0.01$) and beta 3 -/- animals consistently induced more angiogenesis than their wild-type littermates.

Figure 3.5: Matrigel Plugs containing VEGF induce more angiogenesis in Beta 3-deficient Animals

Sections of each Matrigel plug stained by H&E were examined by light microscopy and the number of blood vessels from 5-10 high power fields were counted and averaged. *In vivo* neovascularization was enhanced in β3-/- mice at all concentrations of VEGF tested. The difference between the mean percentage value of β3-/-and control animals was significant (p= 0.04 (30ng/ml), 0.007 (60ng/ml), 0.002 (250ng/ml)). Each column represents the mean ± S.E. of 3-4 plugs/group per genotype.


35
30
25
20
15
10
5
0
Vessels/hpf
PBS
30ng/ml VEGF
60ng/ml VEGF
250ng/ml VEGF
wild-type
beta 3 -/-

Section 3.3: Discussion

In this chapter, I have extended our recently published data (Reynolds et al., 2002) on the enhanced angiogenesis which occurs in systems lacking beta 3 integrin. In addition to showing increased vessel density in tumors, our publication presents data from two different angiogenesis assays in which a single concentration of VEGF was tested for its ability to induce an angiogenic response in wild-type and beta 3-deficient animals. My data are consistent with an enhanced angiogenic response of β3-null aortic rings and in Matrigel implants in β3-/- animals. Increasing concentrations of VEGF did not consistently produce further increases in the angiogenic response. Low doses (10ng/ml, 30ng/ml, 50ng/ml) produced dose-dependent enhancement but higher concentrations did not, but these results could be expected because in pathological conditions of chronic hypoxia in fact VEGF is no longer upregulated by hypoxia (Levy, 1999). This suggests that certain responses can be sensitized in EC and thresholds in maximum angiogenesic response can be reach. These data support the conclusion that increased levels of VEGFR-2 are expressed in beta 3-null animals and thus there is the potential for increased mitogenic potential in these cells.

Determination that this effect is dose-dependent, to an extent, may help to realize which of the changes in VEGFR-2 are in fact primary to the angiogenic phenotype seen. The nature of expression of VEGF, released into solution and also bound to matrix, makes it difficult to know what are physiological relevant concentrations. It is likely that at sites of MMP activity, localized increases of available VEGF will be found. In my studies, although wild-type samples were approaching an equal extent of angiogenesis as

the β3-/- samples, in the conditions tested, they were incapable of reaching β3 maximal levels. This could mean that the increased VEGFR-2 levels on the integrin-deficient MLEC is significant for a maximal response. At lower concentrations of VEGF however, it is likely that wild-type cells express sufficient levels of VEGFR-2, but β3 is experiencing a higher effective dose of VEGF due to an upregulation of this protein as well as its receptor. Unpublished data from collaborators have shown in ELISA studies that beta 3-deficient mice do have higher levels of circulating VEGF (Reynolds, L and Robinson, S, pers.comm.).

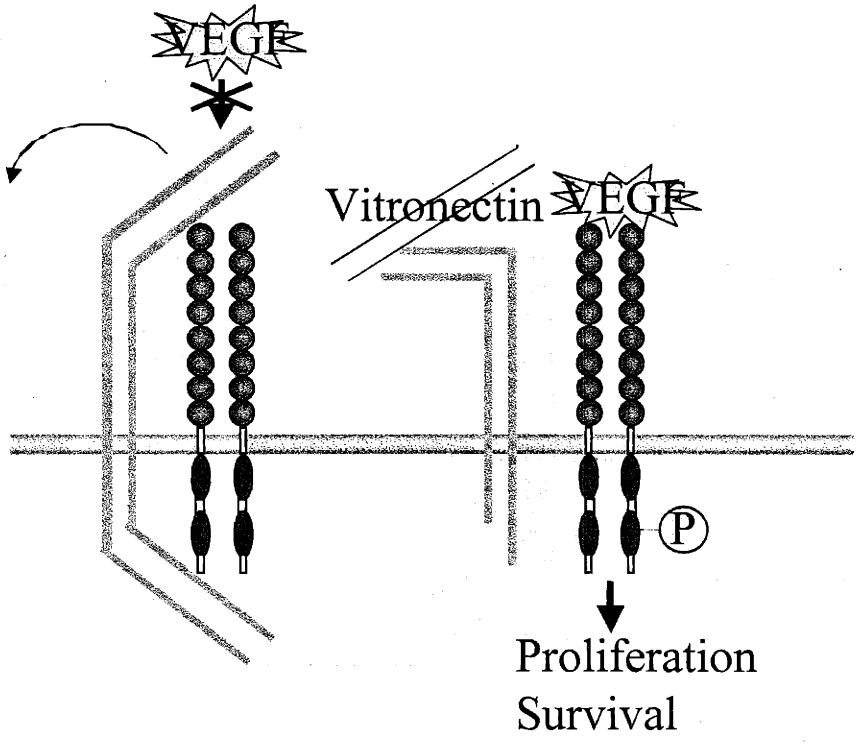
At least one of two possible non-mutually exclusive mechanisms is responsible for these changes in VEGF and VEGFR-2 expression. . If, as interference studies have suggested, β3 integrin is a critical proangiogenic molecule, early in development the organism may compensate genetically for its absence by increasing the basal levels of another proangiogenic growth factor and ligand providing for more robust signaling. On the other hand, the synergy between integrin-mediated adhesion and growth factor stimulation to produce mitogenic responses has been well documented and thus could help to explain the connection. Multiple mechanism of a functional regulation of this pair of molecules by β3 can also be suggested. Beta 3 may normally regulate VEGF and VEGFR-2 function through regulation of downstream signaling. The absence of β3 therefore may release this negative control to give the same apparent result. This integrin may also physically interact on the cell surface with VEGFR-2 and likewise inhibit its activity (Soldi et al., 1999). As I discussed in Chapter 1:Introduction the potential interaction of MMPs and αvβ3 integrin (Brooks et al., 1996) and the function of MMP to release matrix bound VEGF (Bergers et al., 2000) may be significant. The absence of

integrin may change the dynamics of MMP release of VEGF. However this is unlikely inlight of the results of Brooks et al. (Brooks et al., 1998) suggesting that MMP activity, a proangiogenic activity, is increased by binding to αvβ3 and in the absence of such binding MMP activity can be inhibited. They show this inhibition by the addition of PEX which binds αvβ3 and inhibits the binding. In the absence of β3 therefore one would assume an inhibition of VEGF levels would occur due to the inability of MMP and beta 3 to interact. More investigation is necessary to truly understand the regulation and importance of MMP activity in beta 3 deficient animals.

This possible physical association of αvβ3 and VEGFR-2 could be either direct or indirect as shown by Borges or Soldi (Borges et al., 2000; Soldi et al., 1999). In fact, αvβ3 but not αvβ5 was co-immunoprecipitated with VEGFR-2 from human endothelial cells. Both studies introduced VN, the ECM ligand for αvβ3, into the experiment which I believe may contribute to the results they have found. Borges et al. observed that when cells were attached to VN, VEGFR-2 was able to stimulate proliferation and migration. Soldi found the same to be true and added VN binding increased phosphorylation of VEGFR-2. It was in fact phosphorylated-VEGFR-2 that was co- immunoprecipitated with αvβ3 integrin.

Figure 3.6: Model of αvβ3 and VEGFR-2 interaction

In the model depicted, αvβ3 inhibits VEGF:VEGFR-2 signaling when unliganded. VN ligation releases this inhibition, increases VEGFR-2 phosphorylation and promotes cell survival. It is unclear if the inhibition is extracellular or intracellular, however, I would suggest that it may be extracellular since VEGF is capable of increasing VN binding perhaps by competing for binding to VEGFR-2 and freeing αvβ3 to bind ligand.



I don't think it is possible to conclude any hard and fast rules about this particular interaction, but that it is more likely just one of many ways angiogenesis can be controlled in the context of many other signals. In the reverse direction, Byzova et al have shown that VEGF can increase cell adhesion to vitronectin through αvβ3 (Byzova et al., 2000). It is not clear what state αvβ3 may rest in but in the presence of some level of VEGF, cell adhesion and migration onto VN can be enhanced while binding of VN may promote the survival of cells through VEGF signaling. Presumably the ligation of

αvβ3 can be shifted from VEGFR-2 to VN and back dependent on environmental events in what appears to be a balancing act between pro- and anti-angiogenic signals.

The principle of integrins mediating growth factor signaling is not a new one. The classic set of experiments looking at vascular integrins αvβ3 and αvβ5 integrin have already been mentioned. Basically the induction of angiogenesis by bFGF required αvβ3 to be free of antagonist and the induction by VEGF required αvβ5 to be free of antibody antagonist (Friedlander et al., 1995). These studies were done in fairly complex environments (cornea and CAM) so the ligand-binding state of the integrin not being antagonized is not evident. If αvβ5 engaged in a similar bi-directional interaction with FGFR an imbalance in free ligand could generate exactly the opposite interpretation of the data than what has been accepted. That is, engagement of αvβ5 is required for bFGF signaling and ligand binding of αvβ3 is required for VEGF signaling, which is consistent with the conclusion drawn by Soldi and Borges. Zheng and Clemmons reported that ligand occupancy of αvβ3 by VN or TSP-1 enhances IGF signal whereas antagonists which block occupancy of this receptor block IGF signaling (Zheng and Clemmons, 1998). Attachment of αvβ3 to vitronectin or tenascin-C has also been shown to enhance EGF phosphorylation in smooth muscle cells (Jones et al., 1997). Platelet derived growth factor receptor β (PDGFR-β) phosphorylation was shown to be upregulated when αvβ3 integrin is engaged by VN (Schneller et al., 1997). The common theme of these studies is that the effects on growth factors by integrins are realized only in the presence of ligand binding and generally a specific ligand. The changes in cell responsiveness to VEGF in the β3 -/- animals indicate that there is some cross talk that has now been lost and generated an increase in pathological angiogenesis.

Several possible experiments could further elucidate the relationship between αvβ3 and VEGFR-2. An alternative strategy for abolishing protein expression is to target the mRNA of interest by introducing antisense RNA constructs, DNAzymes or ribozymes, therefore disrupting protein translation (Jen and Gewirtz, 2000). RNA interference, or the introduction of a double stranded RNA also effectively cause gene silencing in a nearly catalytic fashion (Nishikura, 2001). Although a remote possibility of compensation does exist at any time given the extended time course required for many angiogenesis assays, this method would likely ensure that "normal" development had taken place and allow for targeted disruption at a given point in time for a given assay.

Other genetically modified cells have been generated which could also be used to test cellular responses to VEGF and bFGF. To clarify the results of Friedlander et al., one could examine the effects of bFGF treatment in VEGFR-2-deficient cells. If the angiogenic stimulus which is abrogated by αvβ3 antagonists is strictly due to a bFGF pathway and not the subsequent upregulation of the VEGF pathway one would expect the same order of inhibition in VEGFR-2-/- cells.

Alternative hypotheses which may be consistent with results from this chapter will be discussed in the final chapter in the context of additional data. I will discuss crosstalk and transdominant inhibition in greater length as well as generate novel hypotheses on β3 function.

Chapter 4: Genetic Analysis of Tumstatin Function

Section 4.1: Introduction

The pro-angiogenic stimulus in vessel development is balanced by negative regulators and modulators, and this chapter will discuss this interplay.

Evidence for potential anti-tumor agents existing within other molecules has been mounting and as discussed in the Introduction, proteolytic fragments of endogenous molecules constitute a broad group of antiangiogenic compounds. Alpha chains of Type IV collagen are apparently a hotbed for antiangiogenic activity. Of 6 alpha chains, three, $\alpha 1$, $\alpha 2$, and $\alpha 3$ fortuitously have been identified to have significant inhibitory potential. Sequence analysis showed that $\alpha 5$ (IV) NC1 varies only by 3 amino acids from $\alpha 3$(IV)NC1 but has no activity (see Fig 4.2). Synthetic peptides (amino acids 185-203) with sequence derived from NC1 domain of the alpha 3 chain of type IV collagen (3(IV)NC1) were shown to inhibit the proliferation of melanoma cells in vitro (Han et al., 1997) and were found to bind to $\alpha v\beta 3$ integrin and CD47/IAP(Shahan et al., 1999). Recently, our collaborators in the Kalluri group at Beth Isreal Deaconess Medical Center at Harvard University, showed that vascular basement membrane-derived 3(IV)NC1, termed "tumstatin," possessed anti-angiogenic activity (Maeshima et al., 2000b; Maeshima et al., 2001a; Maeshima et al., 2001b; Petitclerc et al., 2000).

In attempts to identify a possible cellular target of tumstatin, they limited their search to molecules, such as integrin $\alpha v\beta 3$, upregulated on proliferating endothelial cells. Despite recent evidence questioning the extent and significance of upregulation (Singh et al., 2000), several studies report $\alpha v\beta 3$ as a marker of angiogenic vessels and a molecule

which plays a critical role in this process as well as in the promotion of endothelial cell survival (Brooks et al., 1994a; Brooks et al., 1994b). Studies, in addition to those of Kalluri and colleagues, have indicated that the 3(IV)NC1 domain contained sequence which could bind αvβ3 (Shahan et al., 1999). Maeshima, Colorado and Kalluri went on to show that in fact there are 2 distinct fragments that bind αvβ3 found in the NC1 domain and both are RGD-independent. They provide evidence that the N terminal sequence, in which active antiangiogenic peptide sequences lie, can bind both endothelial cells and melanoma cells yet only has the ability to inhibit proliferation of endothelial cells. Whereas the C-terminal sequence identified by Shahan also binds to both endothelial cells and melanoma cells yet can only inhibit the proliferation of the melanoma cells with no effect on endothelial cells(Maeshima et al., 2000a). These data suggest an interesting dual specificity of this possibly naturally occurring NC1 domain fragment of CollIV.

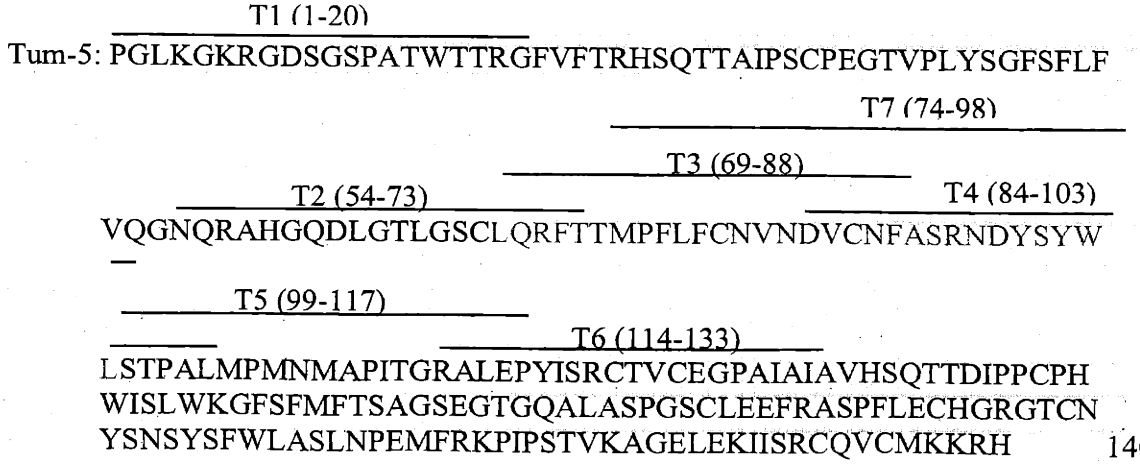
They suggest that as matrix is remodeled, this molecule has the abilty to inhibit proliferation of the tumor cells as well as the cells that feed them. Pursuit of studies exclusively examining the N-terminal T7 peptide fragment shall help distinguish the importance of the endothelial cell survival to the survival of the tumor mass. Tumstatin was found to bind αvβ3 integrin in competitive proliferation assays, cell attachment assays and by ELISA. This interaction was RGD-independent, insofar as αvβ3 was still capable of engaging ligand in the presence of tumstatin (Maeshima et al., 2000a).

Proliferation assays with bovine lung endothelial cells were used to analyze peptides derived from the NC1 domain of the alpha 3 chain of Type IV Collagen and several active peptides were determined (Maeshima et al., 2000a; Maeshima et al.,

2000b; Maeshima et al., 2001a; Maeshima et al., 2001b). The active antiangiogenic sequence of tumstatin was characterized initially, by deletion mutagenesis , as the 54-132-amino acid anti-angiogenic domain, tum-5 (Maeshima et al., 2000b). Tum-5 inhibited neovascularization and tumor growth in vivo using both Matrigel Plug and PC-3 xenograft tumor graft models of angiogenesis (Maeshima et al., 2001a). Tum-5 also inhibited angiogenesis through αvβ3 integrin interaction on endothelial cells as previously shown for the full-length tumstatin. Additionally, Tum-5 interaction with αvβ3 integrin is independent of vitronectin or fibronectin binding to endothelial cells, creating the possibility that there are additional "ligand" binding sites on this integrin which have downstream effects. Since Maeshima and others showed that no secondary structure was necessary for tum-5 activity (Maeshima et al., 2001b), 20-25 amino acid overlapping peptide fragments were synthesized to determine the smallest region needed for antiangiogenic activity (see Fig. 4.1).

Figure 4.1: Tumstatin derived peptide sequences

Tum-5 was isolated in deletion studies from the NC1 domain of α3 IV Collagen for its ability to inhibit endothelial cell proliferation. The following depicts the sequence and alignment of peptide fragments of Tum-5 used or referred to in my studies of tumstatin derivatives. Residues not in black represent those which belong to peptides that exhibit antiangiogenic activity.



Assays similar to those used initially to characterize the antiangiogenic potential of the α3(IV) NC1 domain were carried out in C-PAE cells and determined T3 to possess the greatest anti-proliferative activity (Maeshima et al., 2001b). Cell attachment and competitive proliferation assays showed that preincubation of T3 with αvβ3 protein was able to abrogate the inhibitory effects of T3. T4 exhibited weak binding to αvβ3 and promoted weak endothelial cell attachment. Modifications of T3 to include 10 amino acids of T4 yielded T7 and restored the activity of all antiangiogenic properties to an ED50 of 1μM, a level equivalent to full-length tumstatin and tum-5. Further modifications were made to improved solubility and purification again with no changes in antiproliferative activity.

Figure 4.2: Collagen NC1 Peptide Fragments Sequence Alignment.

T3, T4, and T7 are tumstatin derivatives used in my studies as inhibitors. T8 has been modified for enhanced solubility. T7A5 is the sequence of the analogous region of the NC1 domain of α5 IV Collagen. Note only 3 amino acid substitutions have rendered this fragment unable to inhibit endothelial cell proliferation or angiogenesis.

```
      69                  84                  96
T3:   LQRFTTMPFLFCNVNDVCNF
T4:                       DVCNFASRNDYSYWLSTPAL
T7:         TMPFLFCNVNDVCNFASRNDYSYWL
T8:   KQRFTTMPFLFCNVNDVCNFASRNDYS
T7A5:       TMPFMFCNINNVCNFASRNDYSYWL
```

In an attempt to define the mechanism of the cellular inhibition, signaling cascades were examined and due to a concomitant cell death phenotype observed in the presence of tumstatin, protein synthesis inhibition was examined.

Rapamycin significantly affects the translation of a subset of mRNAs involved in cell-cycle progression. The cellular target of rapamycin is mTOR (mammalian target of

rapamycin, also named FRAP/RAFT1)(Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995), a member of the ATM-related kinase family, homologous to phosphatidylinositol kinases (Cimprich et al., 1996). mTOR is required for mitogenic regulation of p70 S6k (Brown et al., 1995; Chou and Blenis, 1995) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1, also known as PHAS-I) (Hu et al., 1994; Kleijn et al., 1996), both of which are involved in the regulation of translation initiation. 4E-BP1binds eukaryotic initiation factor 4E (eIF4E) thus inhibiting 5' mRNA cap-binding-complex formation and protein translation. Upon mitogenic stimulation, phosphorylation of 4E-BP1, by mTOR, disrupts eIF4E binding and frees eIF4E to initiate translation (Pause et al., 1994). The initiation factor eIF4E is part of a complex of initiation factors (including eIF4A, eIF4B and eIF4G) that assemble on the mRNA cap site and possess an RNA helicase activity. The eIF4E binds directly to the N7-methylguanosine cap structure of mRNA, providing docking for eIF4A, B, and G subunits on the 5' end of the mRNA. Two binding proteins, 4E-BPs, when dephosphorylated, bind directly to eIF4E and competitively inhibit association of eIF4G with eIF4E and presumably block helicase activity (Pause et al., 1994). Stimulation of the cells results in phosphorylation of 4E-BPs, release of the inhibition and translation.

The findings in wild-type cells and organisms suggesting $\alpha v\beta 3$ plays a role in the inhibition of angiogenesis by tumstatin could be best confirmed by analysis in a beta 3 deficient background. In this chapter, I will provide evidence that in the absence of beta 3 integrin, tumstatin is ineffective in all capacities. Unlike wild-type MLEC, MLEC isolated from beta 3-deficient animals were not growth-inhibited by incubation with tumstatin and no inhibition of protein synthesis was seen. *In vivo*, tumstatin could not

inhibit VEGF-induced angiogenesis in a Matrigel Plug assay in beta 3-null animals. These data provide genetic proof that beta 3 integrin is necessary for tumstatin activity.

Section 4.2: Tumstatin Results:

The specificity of tumstatin for αvβ3 was definitively tested with the use of animals and cells deficient for this integrin. In vitro assays, which will be described in this chapter, utilized the mouse lung endothelial cells isolated and purified from beta 3-deficient animals in comparison with wild-type MLEC to assay inhibition of proliferation and the dependence of tumstatin activity on the presence of alpha v beta 3 on endothelial cells. A majority of these assays were done jointly with Yohei Maeshima, BIDMC, Harvard Medical School and the [^{35}S]-Methionine incorporation was carried out by Sudhakar Akullapalli (BIDMC) on cells I had prepared and cultured. The results strongly implicate the use of this protein domain as a promising drug candidate in the treatment of diseases dependent on angiogenesis.

Endothelium-Specific Anti-proliferative effect

Mouse lung endothelial cells isolated from wild-type and beta 3 integrin-deficient animals were treated with tumstatin and assayed for proliferation. Endothelial cells synchronized by contact inhibition and replated at subconfluent concentrations were treated in exponential growth phase with peptides and assayed for proliferation measured by radioactive thymidine incorporation. Treatment of wild-type but not beta 3-deficient endothelial cells with active synthetic derivatives of tumstatin showed significant inhibition of endothelial cell proliferation as measured by [^{3}H]- thymidine incorporation (Fig.4.1). Peptides which inhibit CPAE proliferation (see Introduction), tum-5 (50 μg/ml), T3 and T7 (10 μg/ml), showed comparable inhibition in wild-type MLEC

whereas control peptide T1, which has shown no activity with any cells tested, failed to inhibit wild-type proliferation (Fig. 4.3). Treatment resulted in 50% less [^{3}H]- thymidine incorporation after a 24 hour incubation. Control NC1 domain of Type XVIII Collagen, endostatin (100 μg/ml), inhibits both wild-type and beta 3-deficient endothelial cell proliferation as expected (O'Reilly et al., 1997). The anti-proliferative effect by T7 was shown to be dose-dependent, assayed both by methylene blue staining (Fig 4.4) and 3H thymidine incorporation (data not shown). The ED50 of this inhibition is approximately 2.5μM.

Fibroblasts isolated from day 17 wild-type embryos express $\alpha v\beta 3$ integrin in culture (Hodivala-Dilke et al., 1999). Mouse embryonic fibroblasts (MEFs) from wild-type animals treated with peptides which inhibit proliferation of MLEC expressing $\alpha v\beta 3$ showed no inhibition of proliferation. As expected, treatment of MEFs from animals lacking beta 3 integrin expression failed to inhibit their proliferation. Endostatin, which inhibits proliferation of endothelial cells regardless of beta 3 integrin expression, had no effect on the proliferation of fibroblasts (Fig. 4.5).

The ability to inhibit proliferation of endothelial cells was compared with a sequences of NCI domains of various alpha chains of Type IV Collagen. The paucity of inhibitory activity of alpha 5 chain of Type IV collagen and the high level of homology in the minimal region determined to carry tumstatin activity (T7), led to the synthesis of a T7 mutant containing only 3 amino acid substitutions representing the differences between the alpha 3 chain and the alpha 5 chain. This mutant failed to inhibit the proliferation of even wild-type MLEC and likewise had no activity against beta 3 integrin deficient endothelial cells (Fig 4.6). Again no inhibition of MEF proliferation was seen

regardless of genotype (Fig. 4.7). This result establishes the specificity of T7 (and tumstatin) for endothelial cells expressing beta 3 integrin.

Figure 4.3: Inhibition of Endothelial Cell Proliferation by Tumstatin

After treatment (T1, T3, and T7: 10 μg/ml, tum-5: 50 μg/ml, endostatin: 100 μg/ml), [^{3}H]-thymidine incorporation was measured as described in methods (Method 6.10). Tumstatin derivatives inhibited serum- (20% fetal bovine serum) stimulated proliferation of mouse lung endothelial cells (MLEC) expressing $\alpha v\beta 3$. No inhibition of proliferation is seen in MLEC deficient in $\alpha v\beta 3$.

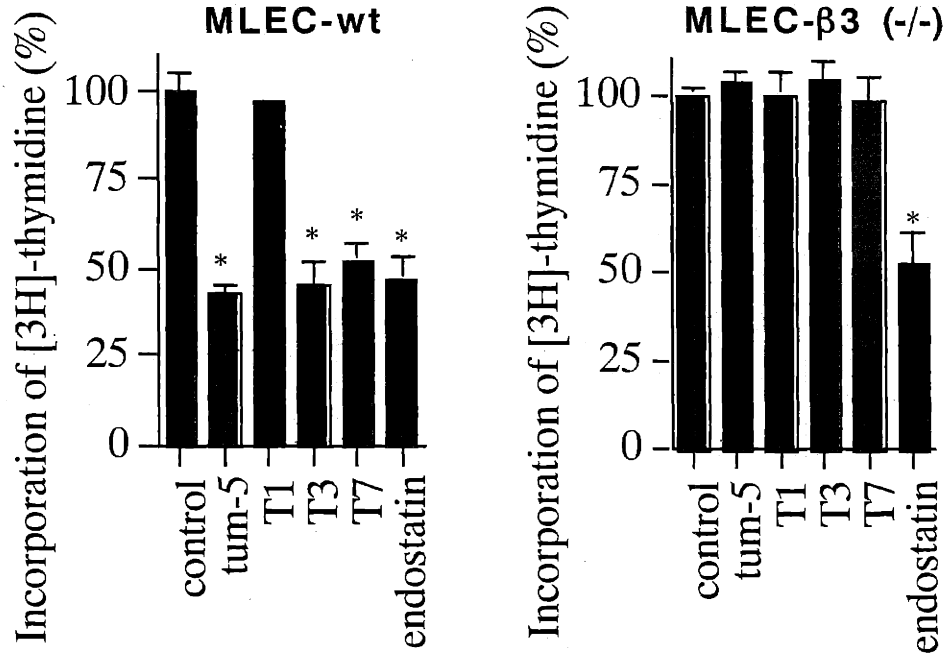


Figure 4.4: Inhibition of MLEC Proliferation by T7 peptide is Dose Dependent

After treatment (T7: 5μM, 2.5μM, 0.5μM), methylene blue staining of cells was measured as described in methods (Method 6.9). T7 inhibited serum- (20% fetal bovine serum) stimulated proliferation of wild-type MLEC expressing αvβ3 in a dose dependent manner.

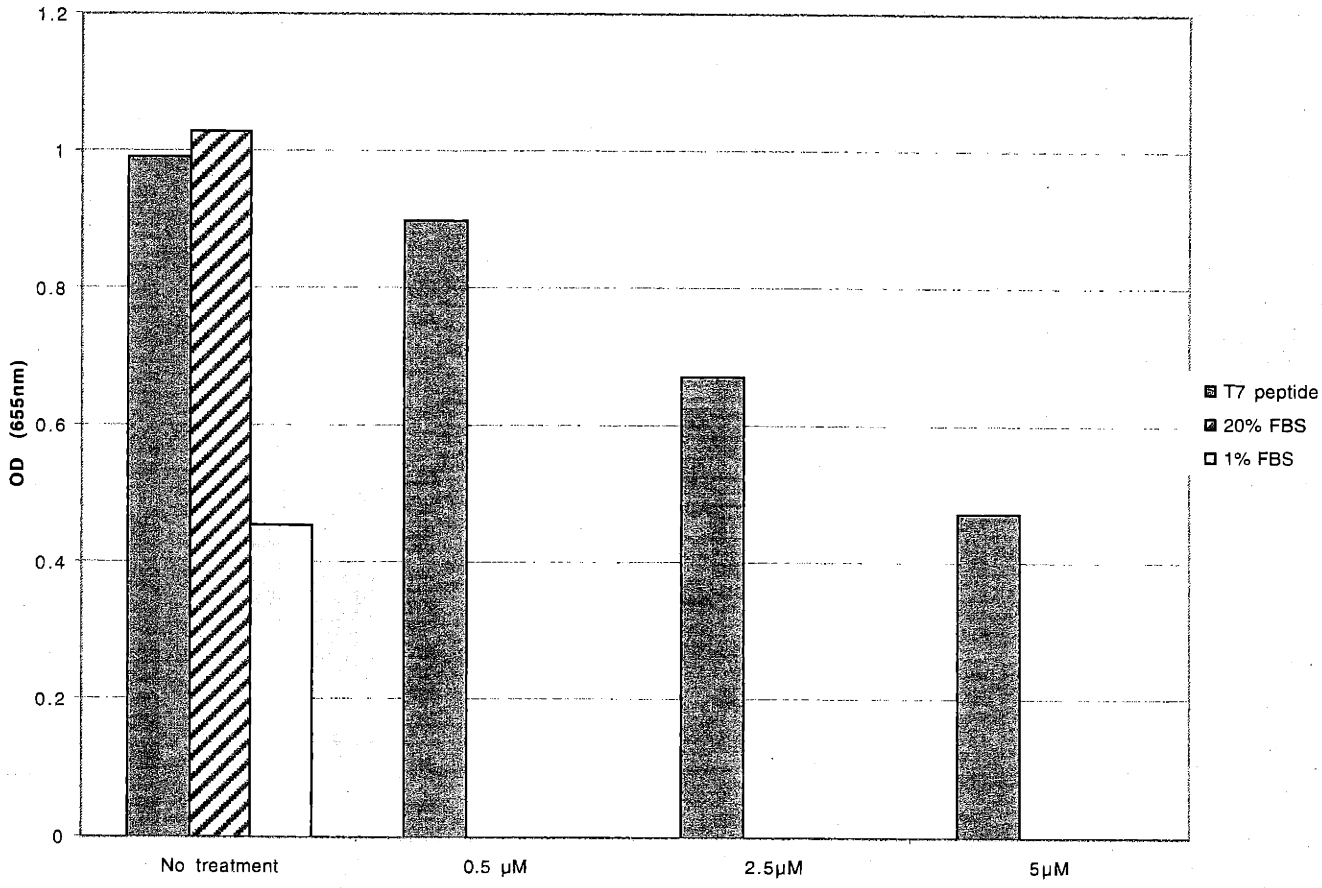


Figure 4.5: Lack of Inhibition of MEF Proliferation by Tumstatin

After treatment (T1, T3, and T7: 10 μg/ml, tum-5: 50 μg/ml, endostatin: 100 μg/ml), [^{3}H]-thymidine incorporation was measured as described in methods (Method 6.10). Tumstatin derivatives failed to inhibit serum- (10% fetal bovine serum) stimulated proliferation of mouse embryonic fibroblast (MEFs) expressing $\alpha v\beta 3$. Additionally, no inhibition of proliferation is seen in MEFs deficient in $\alpha v\beta 3$.

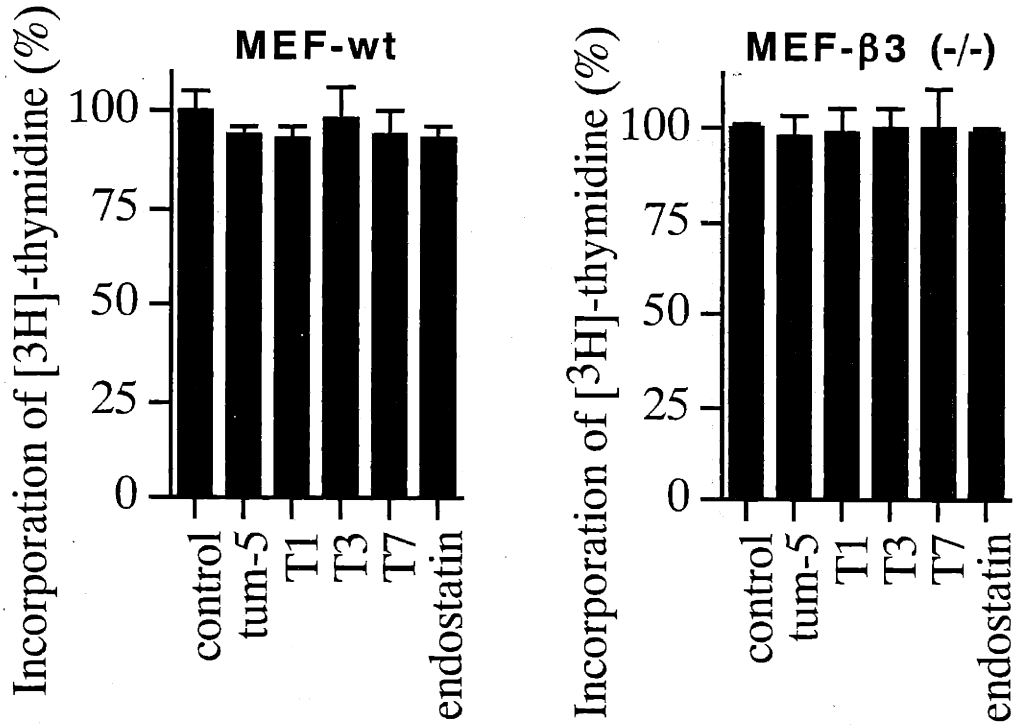


Figure 4.6: Inhibition of MLEC proliferation by Tumstatin: No effect with T7 Alpha 5 mutant

After treatment with peptides (tum-5: 50 μg/ml , T3 and T7: 10 μg/ml, T7 alpha 5 mutant: 10 μg/ml endostatin: 100 μg/ml), [^{3}H]-thymidine incorporation was measured as described in methods (Method 6.10). Again, tumstatin derivatives inhibit serum- (20% fetal bovine serum) stimulated proliferation of mouse lung endothelial cells (MLECs) expressing $\alpha v\beta 3$ yet mutations in T7A5 abolish this inhibition in wild-type cells. These changes have no novel effect in MLECs deficient in $\alpha v\beta 3$ and no tumstatin derivatives inhibit proliferation in the absence of $\alpha v\beta 3$.

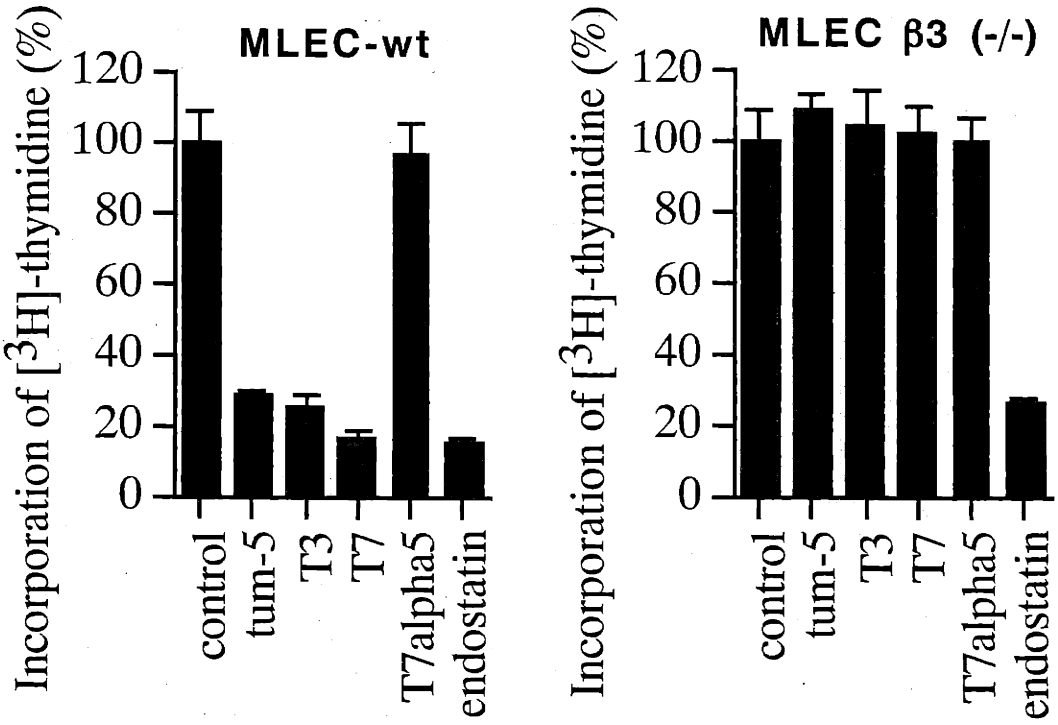
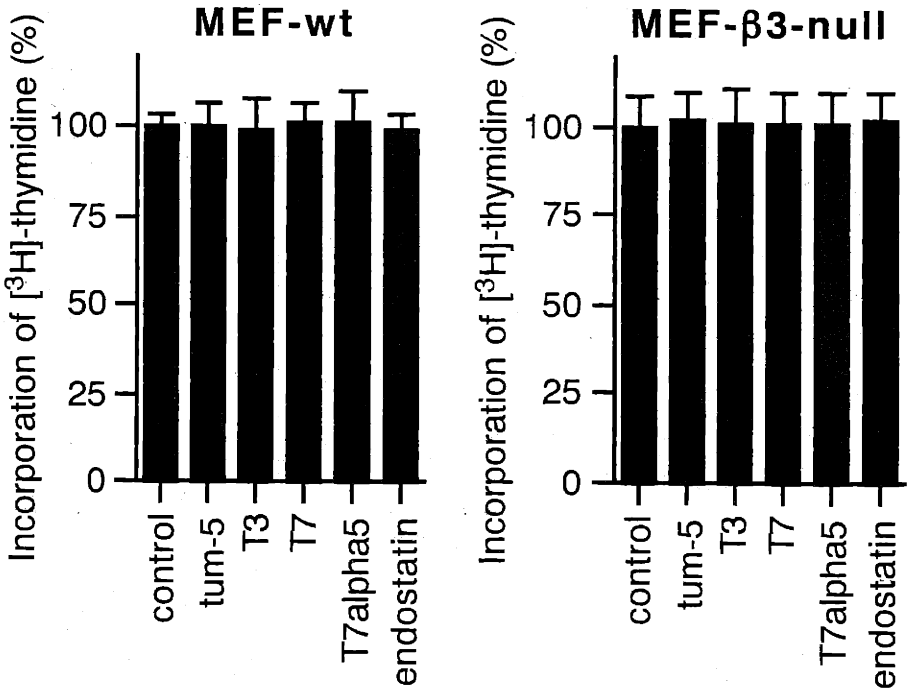


Figure 4.7: Inhibition of MEF proliferation by Tumstatin: No effect with T7A5 mutant

After treatment (tum-5: 50 μg/ml , T3 and T7: 10 μg/ml, T7 alpha 5 mutant: 10 μg/ml endostatin: 100 μg/ml), [^{3}H]-thymidine incorporation was measured as described in methods (Method 6.10). Tumstatin derivatives failed to inhibit serum- (10% fetal bovine serum) stimulated proliferation of mouse embryonic fibroblast (MEFs) expressing $\alpha v\beta 3$. Additionally, no inhibition of proliferation is seen in MEFs deficient in $\alpha v\beta 3$.



Protein Synthesis Inhibition

Total protein synthesis in MLECs from wild-type and beta 3 integrin-deficient animals was measured. Treatment of cells for 21 hours with inhibitors, tum-5 (50 μg/ml), T3 or T7 (10 μg/ml), decreased [^{35}S]- methionine incorporation into TCA-precipitable material in control wild-type cells (β3+/+) but did not have any effect in mouse lung endothelial cells lacking αvβ3 integrin (β3-/-). Rapamycin, a general inhibitor of protein synthesis regulating the downstream activity of mTOR, inhibited protein synthesis in both control and αVβ3 integrin-deficient MLEC. Active peptides showed comparable inhibition of total protein synthesis in wild-type MLEC whereas control mutant peptide T7 alpha 5 (10μg/ml), which has shown no activity with any cells tested, failed to inhibit wild-type protein synthesis (Fig. 4.8). Despite inhibition of endothelial cell proliferation similar to tumstatin, endostatin showed no inhibition of [^{35}S]- methionine incorporation and therefore no inhibition of total protein synthesis of either wild-type or β3– integrin-deficient endothelial cells. Rapamycin was the sole agent tested which inhibited protein synthesis in β3 -/- MLEC; no NC1 domain molecules could inhibit protein synthesis.

In order to establish the specificity of tumstatin for endothelial cells, day 17 mouse embryonic fibroblasts expressing αvβ3 integrin or day 17 MEFs isolated from αvβ3 integrin-deficient animals were also tested in protein synthesis experiments for their responsiveness to tumstatin. Regardless of αvβ3 integrin expression, neither T3 nor T7 peptides inhibited protein synthesis in these MEFs. As with endothelial cells, T7 mutant peptide and endostatin were also ineffective in inhibiting protein synthesis,

whereas rapamycin inhibited protein synthesis in both wild-type and beta 3-deficient cells (Fig. 4.9).

Figure 4.8: Inhibition of total Protein Synthesis by Tumstatin and Derivatives

Endothelial cells (MLEC) from β3-integrin-deficient and wild-type control mice were used to evaluate the effect of tumstatin-derived peptides on total protein synthesis. After treatment with peptide (T3, T7, and mutant T7 peptide: 10 μg/ml, tum-5: 50 μg/ml, endostatin: 100 μg/ml or rapamycin 100 ng/ml), cells were labeled with [^{35}S]-methionine and cell lysates were prepared as described in methods (Method 6.12). Incorporation of [^{35}S]-methionine radioactivity was measured using a liquid scintillation counter. Each column consists of mean+/-SEM of triplicates. Experiment was repeated three times and one representative experiment is shown.

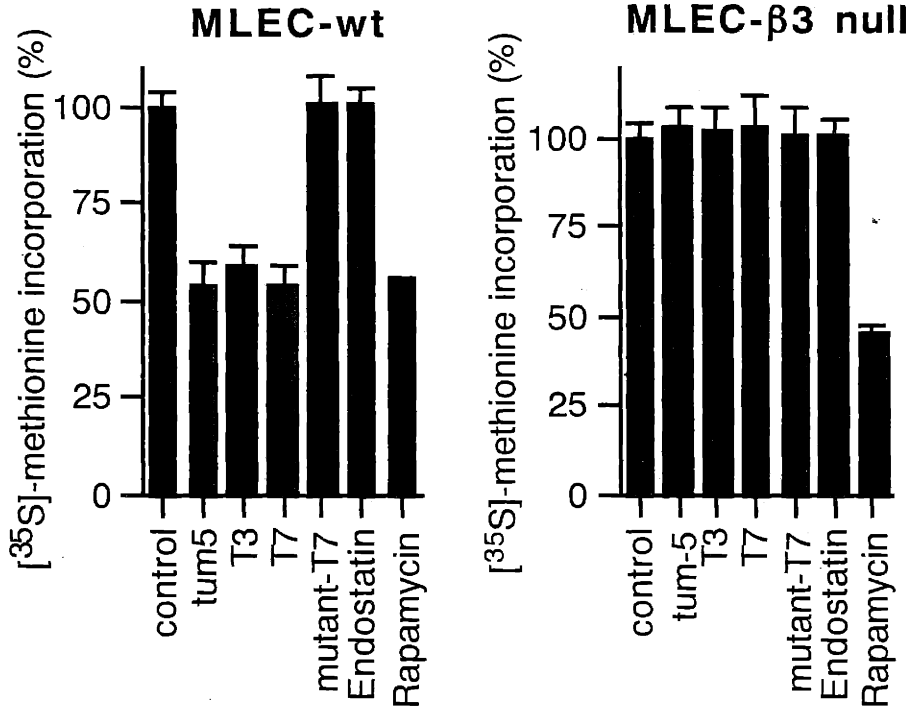
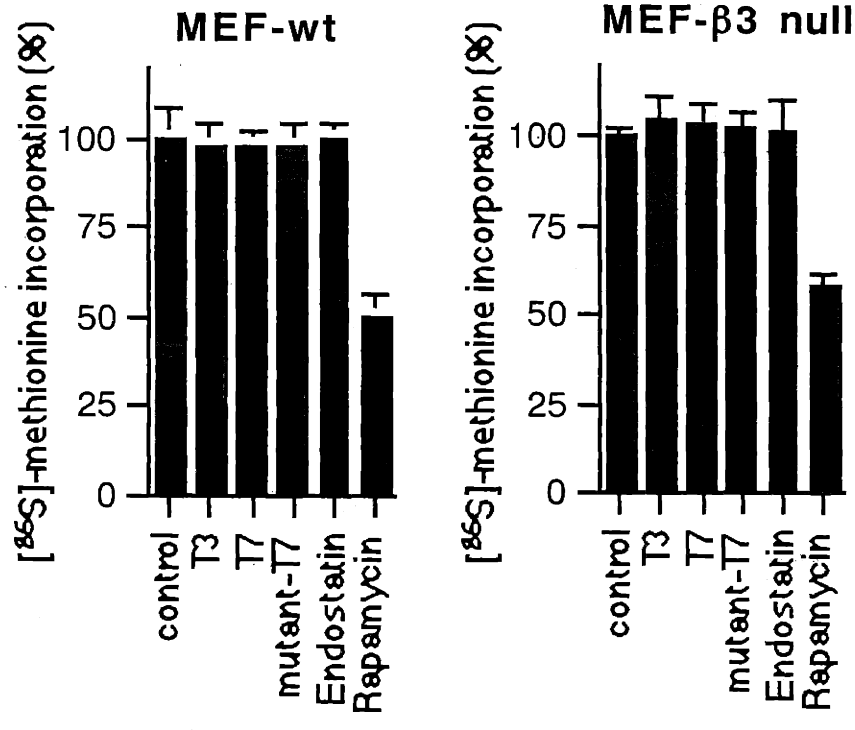


Figure 4.9: No Inhibition of Total Protein Synthesis in MEFs by Tumstatin

Murine embryonic fibroblasts (MEFs) from β3-integrin knockout and wild-type littermate mice were used to evaluate the effect of tumstatin-derived peptides on total protein synthesis. After treatment (T3, T7, and mutant T7 peptide: 10 μg/ml, endostatin: 100 μg/ml or rapamycin 100 ng/ml), cells were labeled with [^{35}S]-methionine and cell lysates were prepared as described in methods (Method 6.12). Incorporation of [^{35}S]-methionine radioactivity was measured using liquid scintillation counter. Each column consists of mean+/-SEM of triplicates. The experiment was repeated three times and one representative experiment is shown.



Protein Synthesis Regulation is CAP-dependent

To explore further the level of inhibition of protein synthesis; transcriptional or translational, reporter constructs were used to assess whether CAP-dependent translation is affected. Cells were either (1) co-transfected with a plasmid containing firefly (*Photinus pyralis*) luciferase gene whose translation is CAP-dependent and a plasmid with constitutive expression of renilla luciferase from the marine organism *Renilla reniformis* or (2) were transfected with a bicistronic plasmid (pcDNA3 -LUC-pol-CAT) alone, containing firefly luciferase gene whose translation is CAP-dependent and chloramphenicol acteyltransferase (CAT) gene preceded by an IRES sequence which will be translated CAP-independently.

When cells are co-transfected, renilla luciferase activity measures transfection efficiency and firefly luciferase measures the relative level of CAP-dependent translation. Due to distinct evolutionary origins, these luciferases have dissimilar enzyme structures and substrate requirements. It is possible to detect each respective activity sequentially in a single sample. This method, however, does not take into consideration any regulation of expression of renilla luciferase outside of translation. There are no post-translational modifications affecting enzymatic activity however. Correcting firefly luciferase readings for transfection (firefly luminescence/renilla luminescence) and reporting this total as a percentage of corrected firefly activity in untreated cells indicates that CAP-dependent translation is specifically inhibited (Fig. 4.10). If the renilla luciferase synthesis is affected transcriptionally the percent inhibition could be inaccurately overrepresented.

To test if any differences could be seen if the control readout is regulated by an absolutely CAP-independent mechanism which directs ribosomes to the start codon for translation initiation a bicistronic plasmid with an IRES was used (Hennecke et al., 2001). Transient transfection of cells with a bicistronic plasmid (pcDNA3 -LUC-pol-CAT) containing firefly luciferase gene with a CAP-binding sequence in the 5' terminus and an internal ribosomal entry site (IRES) derived from 5' untranslated region of poliovirus followed by chloramphenicol acetyltransferase (CAT) gene permits quantification of CAP-dependent and CAP-independent translation of proteins (Beretta et al., 1996; Kumar et al., 2000). Expression of the firefly luciferase is regulated at the translational level and its activity represents CAP-dependent translation whereas expression of the CAT reporter is independent of this regulation and represents CAP-independent translation. CAT activity in all samples remained nearly constant and similar results as above were obtained for CAP-dependent translation of firefly luciferase in wild-type mouse lung endothelial cells. All active derivatives of tumstatin tested, including tum-5, T7, T3 but not mutant T7, inhibited CAP-dependent translation by roughly 40-50%, an amount typical of rapamycin-induced inhibition (Fig. 4.11). Rapamycin treatment of MLEC incidently was shown to enhance translation from the IRES as has been reported previously in other cells (Deffaud and Darlix, 2000). This yields an inaccurate overestimation of protein synthesis inhibition by rapamycin in these cells (Fig 4.11). It is not clear why this effect is not seen in MEFs (Fig. 4.12). There was no inhibition of CAP-dependent protein synthesis in β3 integrin-deficient endothelial cells by any NC1 domain derived peptide yet rapamycin still significantly inhibited luciferase activity in the absence of this integrin.

Consistent with proliferation assays and [^{35}S]- methionine incorporation, there was no inhibitory activity of any peptide tested on mouse embryonic fibroblasts regardless of the expression of αvβ3 integrin on the cell surface. Expression of this integrin is <u>not</u> <u>sufficient</u> for tumstatin or any of its derivatives to inhibit protein synthesis.

Figure 4.10: Inhibition of Protein Synthesis of CAP-dependent reporter in MLEC

Luciferase activity was measured in MLECs transiently transfected with pcDNA3-LUC-pol-CAT and control plasmid, pRL-TK. Cells were treated for 21 hrs in medium containing 10% FBS and tum-5 (50 μg/ml), T3 peptide (10 μg/ml), T7 peptide (10 μg/ml), T7A5 mutant peptide (10 μg/ml), endostatin (100 μg/ml) or rapamycin (100 ng/ml). Total cell extracts were prepared and assayed for luciferase activity. Activity was corrected relative to control renilla luciferase activity. Graphs represent firefly luciferase activity expressed as percent of control. Each column consists of mean+/-SEM of triplicates. These experiments were repeated three times and representative data are shown.

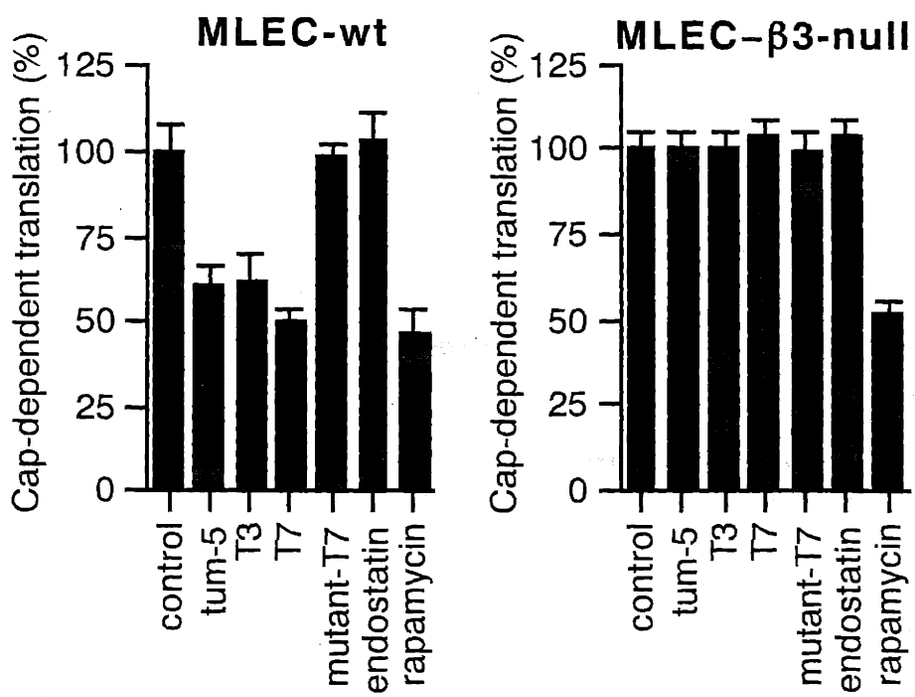
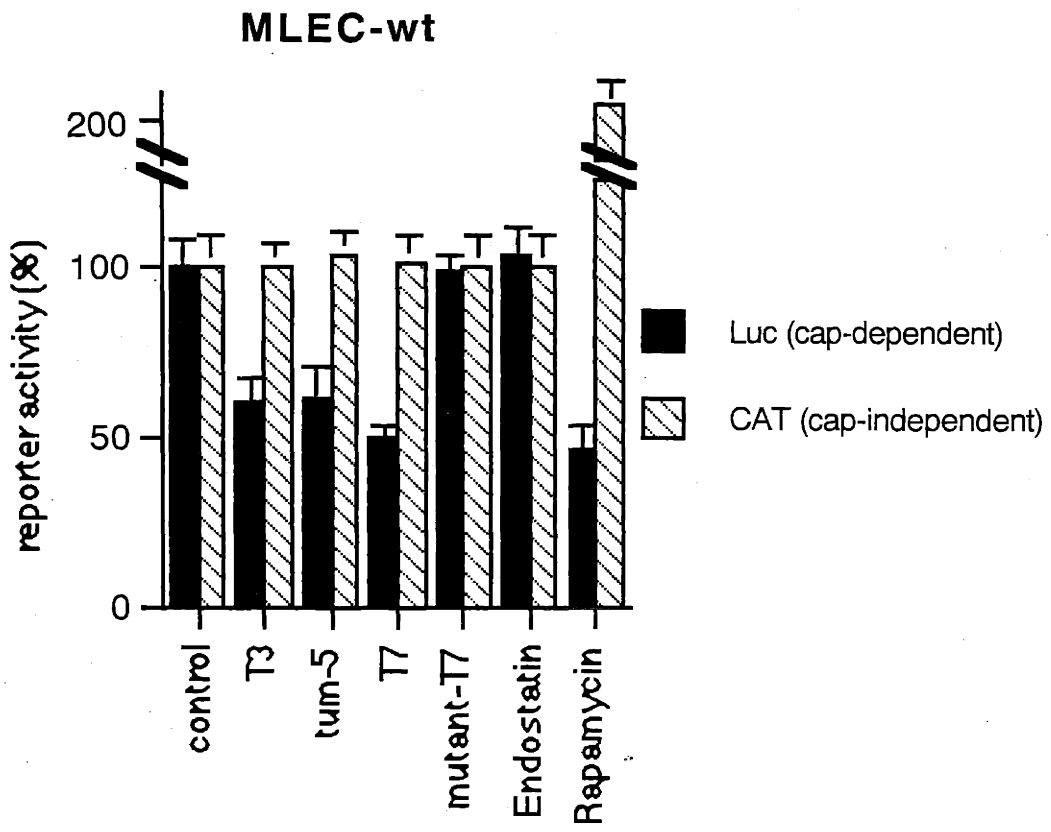


Figure 4.11: CAP-independent Protein Synthesis is unaffected in MLEC

Luciferase activity and CAT activity were measured in lysates of MLECs transiently transfected with pcDNA3-LUC-pol-CAT. Cells were treated for 21 hrs in medium containing 10% FBS and tum-5 (50 μg/ml), T3 peptide (10 μg/ml), T7 peptide (10 μg/ml), T7A5 mutant peptide (10 μg/ml), endostatin (100 μg/ml) or rapamycin (100 ng/ml). Total cell extracts were prepared, divided in half and assayed for luciferase activity or CAT activity. Graphs represent luminometer reading of firefly luciferase activity expressed as percent of control luciferase activity and cpm measured by liquid scintillation counting of butyrylated [^{14}C] chloramphenicol products as a percentage of counts in untreated control. Results in wild-type MLEC are shown in A and beta 3-deficient MLEC are shown in B. Each column consists of mean+/-SEM of triplicates. This experiment was repeated three times and representative data are shown.

A.


MLEC-wt
reporter activity (%)
200
100
50
0
control
T3
tum-5
T7
mutant-T7
Endostatin
Rapamycin
Luc (cap-dependent)
CAT (cap-independent)

B.

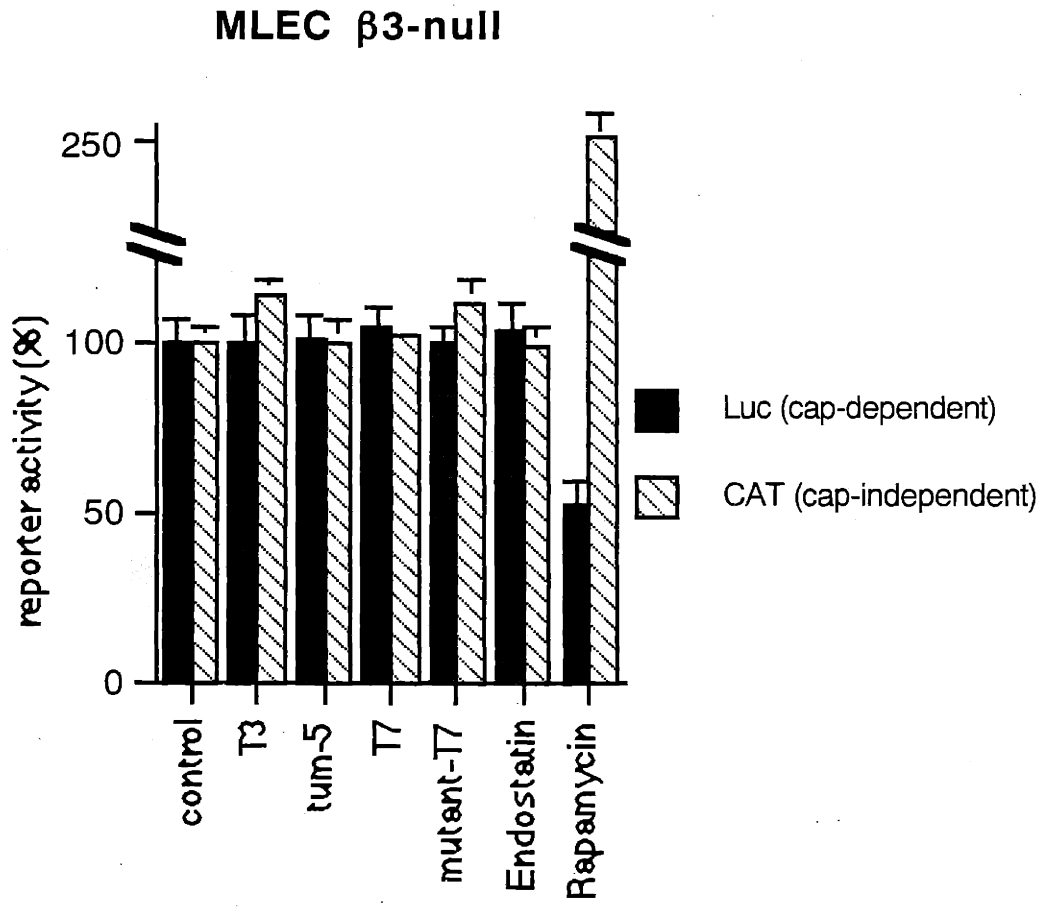
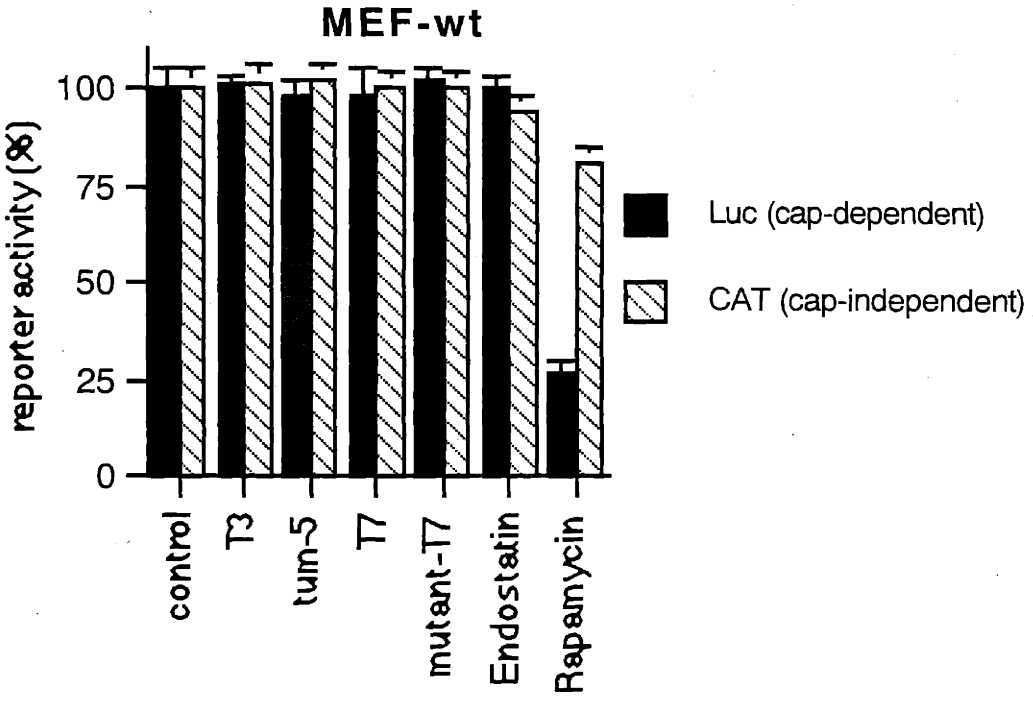
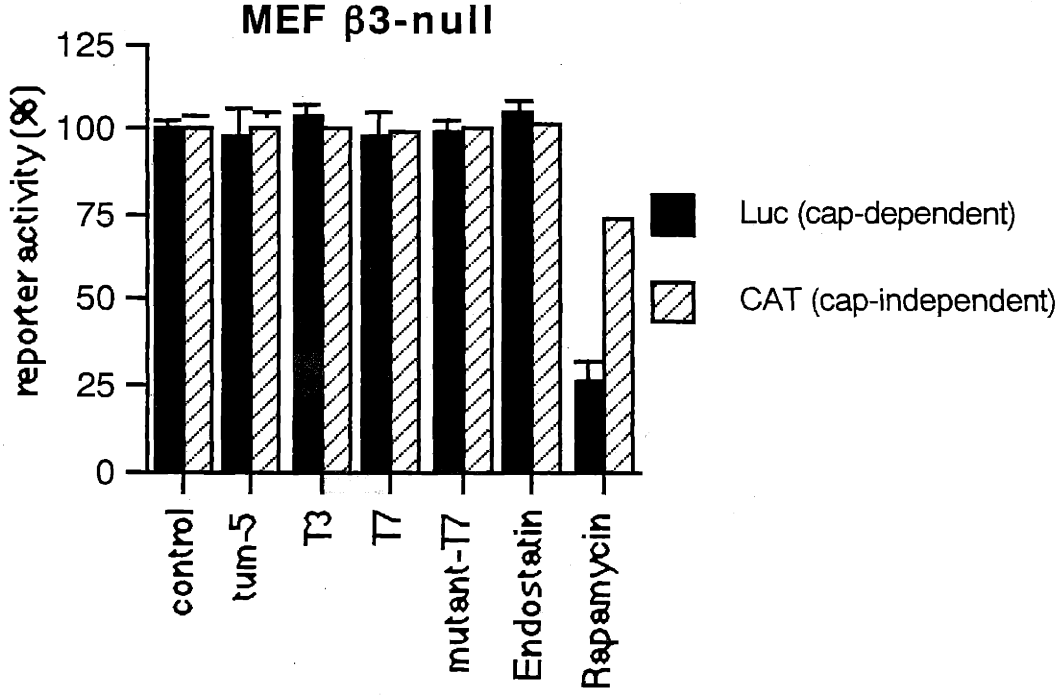

MLEC β3-null
reporter activity (%)
250
100
50
0
control
T3
tum-5
T7
mutant-T7
Endostatin
Rapamycin
Luc (cap-dependent)
CAT (cap-independent)

Figure 4.12: CAP independent Protein Synthesis in MEF is Unaffected

Luciferase activity and CAT activity were measured in lysates of MEFs transiently transfected with pcDNA3-LUC-pol-CAT. Cells were treated for 21 hrs in media containing 10% FCS and tum-5 (50 μg/ml), T3 peptide (10 μg/ml), T7 peptide (10 μg/ml), T7A5 mutant peptide (10 μg/ml), endostatin (100 μg/ml) or rapamycin (100 ng/ml). Total cell extracts were prepared, divided in half and assayed for luciferase activity or CAT activity. Graphs represent luminometer reading of firefly luciferase activity expressed as percent of control luciferase activity and cpm measured by liquid scintillation counting of butyrylated [^{14}C] chloramphenicol products as a percentage of counts in untreated control. Results in wild-type MEFs are shown in A and beta 3-deficient MEFs are shown in B. Each column consists of mean+/-SEM of triplicates. This experiment was repeated three times and representative data are shown.


MEF-wt
reporter activity (%)
100
75
50
25
0
control
T3
tum-5
T7
mutant-T7
Endostatin
Rapamycin
Luc (cap-dependent)
CAT (cap-independent)

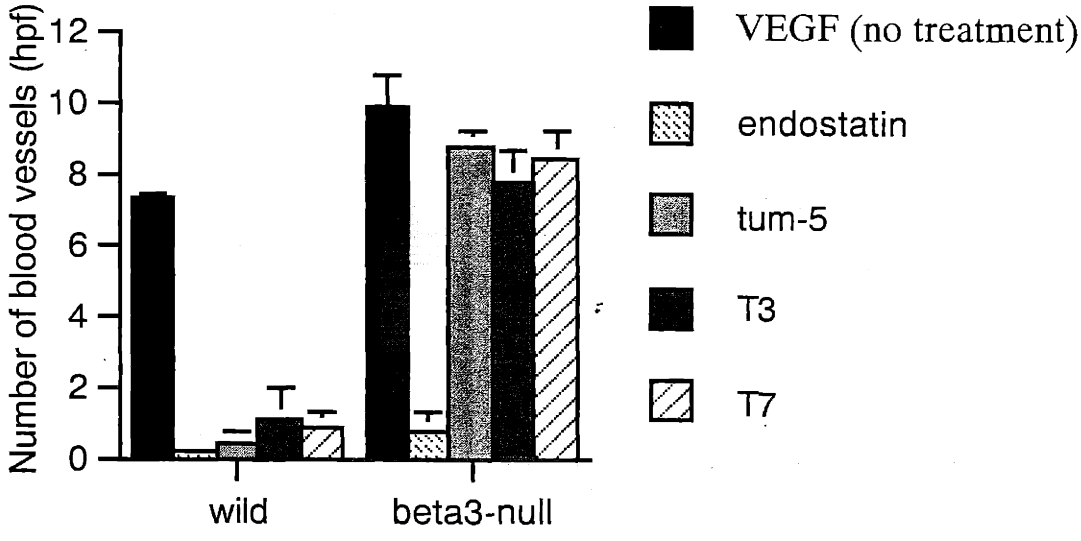

MEF β3-null
reporter activity (%)
125
100
75
50
25
0
control
tum-5
T3
T7
mutant-T7
Endostatin
Rapamycin
Luc (cap-dependent)
CAT (cap-independent)

Matrigel Implants

To evaluate the effects *in vivo* of synthetic tumstatin derivatives on de novo vessel formation, we performed a Matrigel plug assay in mice expressing beta 3 integrin or mice genetically deficient for this integrin. When quantified in a masked fashion, plugs which contain VEGF (50 ng/ml) induce neovascularization in both wild-type and beta 3-null animals. In the absence of growth factor, background levels of vessels were seen (1-2 vessels/hpf). Matrigel was premixed with VEGF and tumstatin derivatives to assess inhibition of growth factor -induced angiogenesis by these synthetic peptides. Tum-5, T3, T7 all inhibit neovascularization of Matrigel plugs to background levels. The number of vessels per high powered field in Matrigel plugs injected into beta 3 integrin-deficient animals remained constant regardless of tumstatin treatment. Endostatin, which inhibits angiogenesis in an $\alpha v\beta 3$ integrin-independent fashion, inhibited neovascularization of Matrigel plugs containing this inhibitor in both wild-type and knock-out animals (Fig. 4.13).

Figure 4.13: Inhibition of Angiogenesis in Matrigel Plug Assay by tumstatin derivatives

Sections of each Matrigel plug stained by H&E were examined by light microscopy and the number of blood vessels from 5-10 high power fields were counted and averaged. Tum-5 (5 μg/ml), T3 peptide (10 μg/ml) and T7 peptide (10 μg/ml) significantly inhibited in vivo neovascularization, as compared with controls (no treatment added). The difference between the mean percentage value of tum-5, T3 or T7 peptide-treated animals and control animals was significant. Each column represents the mean ± S.E. of 3-4 plugs/group.



Section 4.3: Tumstatin Discussion

In this chapter, I have been able to address clearly the specificity of tumstatin derivatives for alpha v beta 3 integrin. Previous data could only provide indirect evidence for the necessity of this integrin through competition binding assays with soluble integrin protein, titrating tumstatin activity prior to incubation with αvβ3 expressing cells (Maeshima et al., 2000a). MLEC genetically deficient for β3 integrin are completely devoid of responsiveness to tumstatin. There is no activity of tumstatin on the endothelial cell proliferation, protein synthesis or neovascularization of Matrigel plug implants in animals or cells lacking beta 3 integrin and thus alpha v beta 3 heterodimer. It is not exclusively the presence of this integrin which provides the necessary components for tumstatin induced protein synthesis inhibition, for embryonic fibroblasts which express αvβ3 integrin fail to respond to tumstatin treatment implicating a second (or more) unknown factor which is necessary for endothelial cell specificity.

Inhibition of endothelial cell proliferation, induction of apoptosis and inhibition of angiogenesis by tumstatin is mediated through the inhibition of protein synthesis. We have shown that the inhibition of protein synthesis by tumstatin requires αvβ3 and is specific for CAP-dependent protein synthesis. Although not presented in this chapter, my recent publication with collaborators include some indication of the downstream effects of tumstatin binding (Maeshima et al., 2002). Tumstatin's interaction with αvβ3 mediates inhibition of the activation of focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3-kinase), protein kinase B (PKB/Akt) and mammalian target of rapamycin

(mTOR), and prevention of the dissociation of eukaryotic initiation factor 4E protein (eIF4E) from 4E-binding protein 1. Tumstatin does not necessarily simply inhibit classic integrin function, as it does not interfere with ligand binding, but we will explore other possibilities as well.

The antiangiogenic activity of tumstatin and its derivatives relies upon the presence of αvβ3 but somehow must also involve additional endothelial-specific factors. Several other signaling components could play a role in the inhibition of endothelial cell proliferation by tumstatin derivatives. It is well known that integrins play a role in mediating growth factor responsiveness (reviewed in Danen and Yamada, 2001). The formation of a focal contact in response to integrin ligation and the recruitment of many signaling molecules is also well studied (Geiger et al., 2001). The effects of tumstatin on integrin signaling through focal adhesion kinase (FAK) were examined and as early as 30 minutes after adhesion to vitronectin, FAK phosphorylation was inhibited in the presence of tumstatin. The inhibition of this proximal signaling event of integrins is highly suggestive that tumstatin is abrogating a survival signal, although that survival signal is not necessarily propagated by ligand attachment to αvβ3. If, as will be discussed at length later, αvβ3 can regulate survival signals from other integrins, the inhibition of FAK phosphorylation may be attributable to tumstatin binding regardless of ligand occupancy. As mentioned in the introduction, integrins can exist in several conformations which modulate their ability to interact with extracellular and intracellular molecules and further experiments would be necessary to determine what state αvβ3 must be in for tumstatin binding and activity. Just as integrins are important for cell proliferation, their role in cell survival and death has also been explored by several

groups including studies exploring the protective role provided by the engagement of αv integrin by vitronectin in the microvasculature (Isik et al., 1998). The ability of several molecules including thrombospondin (Guo et al., 1997), angiostatin (Claesson-Welsh et al., 1998) (Lucas et al., 1998) and transforming growth factor-β (Tsukada et al., 1995) to block angiogenesis can be attributed, at least in part, to their stimulation of cellular apoptosis. Thrombospondin induces DNA fragmentation indicative of apoptosis; angiostatin treatment increases annexin V positivity and TUNEL positivity in vitro cultures of endothelial cells; and TGF-β induces chomatin condensation in HUVECs indicative of apoptotic nuclei. With independent evidence and commonality for either integrins or antiangiogenic agents to impact cell death, collaborators first assessed the activation state of members of the bcl-2 pathway in response to tumstatin treatment. The protein level of bcl-2 was significantly reduced upon T3 treatment of C-PAE and a concomitant increase in caspase 3 activity (3.6 fold) and annexin V staining was observed. Caspase-8, however, was not regulated by tumstatin binding of CPAE (personal comm.Maeshima et al., 2001b).

The expression of αvβ3 is not limited to endothelial cells yet the activity of tumstatin and derivatives is limited to this cell population. There is no evidence as of the writing of this document of what imparts this specificity. An additional endothelial specific molecule is likely involved but other cell specific factors such as affinity, avidity or activation of beta 3 integrin or αvβ3 heterodimer may also be involved. There is no evidence to restrict the identity of this potential additional molecule to an intracellular signaling partner of αvβ3, a co-receptor also expressed on the endothelial cell surface or

even an extracellular molecule which could act as a co-ligand. Several possible molecules appear as likely candidates.

An endothelial specific co-receptor seems likely but could function at several layers to impart a specificity. A clustering effect or simple cooperative binding to raise the effective stoichiometric concentration of tumstatin may be necessary for proper tumstatin function. Upon binding to this co-receptor, tumstatin may trigger certain signaling cascades that act in concert with signaling through $\alpha v\beta 3$ integrin. A second event, a ligand-receptor engagement for example, may necessarily be co-triggered to activate cellular events or processes on which tumstatin signaling impinges leading to downstream inhibition of protein synthesis.

The specificity of VEGFR and its signaling counterpart VEGF for stimulation of endothelial cells, raise a possiblilty these molecules may be involved in tumstatin's endothelial specificity. It is interesting to note that MMP release of VEGF and tumstatin would also be coordinately produced providing circumstantial evidence that both molecules co-exist and at least the formal possibility they could be acting together. Initially my thoughts were to determine if the specificity of action of tumstatin could be seen only in VEGF-treated cells, not bFGF-treated cells, yet it is likely that bFGF will stimulate the VEGF pathway as well (Hata et al., 1999). One would need to test if VEGF molecule is necessary, VEGFR presentation only, or VEGFR function and signaling is the necessary component. To interfere with VEGF access to cells, neutralizing antibodies to VEGF can be added to cells in culture. Any exogenous or endogenous VEGF would no longer be available to assist in the "docking" of tumstatin to endothelial cells if this molecule were important for the specificity. If a dominant

negative mutant of VEGFR were developed this would not eliminate the possibility that non-signaling surface expression is sufficient, yet it would address if VEGF-induced signaling were the endothelial specific component. Since flk-1 deficient animals are not viable it is not possible to isolate primary endothelial cells from them. It may be possible to interfere with surface expression of VEGFR through the use of anti-sense RNA on wild-type cells but even better Kyungchee Choi of Washington University has differentiated Flk-1-deficient embryonic stem cells into endothelial cells as a cell to test our hypotheses. Testing the ability of tumstatin to inhibit these endothelial cells would give definitive evidence if VEGFR is necessary for function.

Integrins also have the capability of associating with many other transmembrane proteins. In fact, there is evidence that cross-talk between $\alpha v\beta 3$ integrin and $\alpha 4\beta 1$ requires integrin associated protein (IAP), thus making it an interesting candidate for a coreceptor for tumstatin. IAP/CD47 cooperates with $\alpha v\beta 3$ in binding TSP-1, another antiangiogenic molecule. Expression of IAP/CD47 is not exclusive for endothelial cells although endothelial cells may express the unique combination of IAP and $\alpha v\beta 3$.

Although the data have not yet defined either the mechanism by which tumstatin manifests its antiangiogenic activity nor even the molecule which imparts endothelial cell specificity, we have added new information to be considered in elucidating the endogenous role of $\alpha v\beta 3$ in angiogenesis.

Chapter 5: Discussion

In order to determine the role of αvβ3 integrin in angiogenesis, we now have several lines of *in vivo* and *in vitro* data which must be incorporated and considered jointly to form a new theory. The absence of defects in developmental angiogenesis and the enhancement of tumor angiogenesis in animals deficient for αvβ3 integrin lead to a current model in which this integrin is a modulator of pathological angiogenesis, not simply an activator. Confusing and ostensibly inconsistent with earlier publications, data generated using genetically deficient animals can actually be joined with prior biochemical interference studies into a more complete understanding of β3 integrin and its function.

The function of endothelial cells considerably contributes to the determination of the angiogenic response. Cultures of isolated endothelial cells from beta 3-deficient animals, consistent with the lack of angiogenic defect in beta 3 –null animals, function apparently normally, proliferating with ease, forming networks *in vitro* and expressing a host of characteristic endothelial markers (see Chapter 2). Biochemical interference studies raised the expectation that endothelial cell function and angiogenesis in the absence of β3 integrin would be significantly impaired, which was not observed. The foundation of studies of endothelial cell function has been based on the idea of alpha v beta 3 as a pro-angiogenic molecule expressed on active endothelium. This presumption is based on and supported by its upregulation on the surface of proliferating endothelial cells and in vessels which are activated, and an apparent downregulation on confluent endothelial cells. Genetic ablation, however, clearly indicates that αvβ3 is not necessary

for formation of the vasculature. This incongruity between genetic and biochemical data involving β3 is atypical for experiments with integrin.

Other vascular integrins expressed on endothelial cells have also been targeted for genetic ablation and the phenotypes of deficient animals are not in conflict with blocking experiments, β1 integrins are a good example. Treatment in various systems with antibodies or peptides interfering with β1 integrin showed a great dependence on this integrin subunit for proper vascular function. Drake et al. (1992) investigated the effects of an antibody to beta 1 integrins (CSAT) using the quail whole embryo system to assay angiogenesis. CSAT was able to inhibit aortic vessel formation at the time of its injection (4-10 somites). Additional studies with β1 antagonists showed inhibition of GF-induced (excluding VEGF-induced) angiogenesis in chick embyo and murine models of tumor angiogenesis (Kim et al., 2000a). Similarly *in vitro*, RGD peptides or antibodies specific for beta 1 integrins inhibit endothelial cell migration and tube formation (Basson et al., 1990; Bauer et al., 1992; Mould et al., 1995).

As predicted by these β1 inhibitor studies, disruption of β1 integrins in the vasculature leads to defects. Alpha 1beta 1, alpha 2 beta 1 and alpha 5 beta 1 integrins are all β1 integrins expressed on vessels with α5β1 contributing most prominently to vessel development as the main FN receptor in endothelial cells. Alpha 1 deficiency is not lethal but, as expected, animals deficient for alpha 1 integrin have defects including impaired tumor vascular development (Gardner et al., 1996; Pozzi et al., 2000). Alpha 5 integrin-null animals die later than animals deficient in their ligand FN, at embryonic day 10-11, but with posterior trunk and significant yolk sac vascular defects (Yang et al., 1993). This phenotype impaired an extensive examination of the role of alpha 5 in *in*

vivo endothelial cell function. Endothelial cells isolated from α5-chimeric animals yielded inconclusive data on the ability of α5-/- to form vascular networks. However, preliminary data suggest alpha 5-null endothelial cells can contribute to vessel formation in the presence of α5-positive endothelial cells in an α5-/- teratocarcinoma in α5 +/+ hosts (Taverna and Hynes, 2001) yet MLEC cultured from chimerae are unable to form networks *in vitro* (Goh, K., personal communication). Analysis of tubule structures in α5-/- embryoid bodies indicates delayed and reduced vessel formation (Francis et al., 2002; Taverna and Hynes, 2001). All of these examples show consistent findings between genetic and biochemical experiments. Several possibilities exist to resolve the incompatibility of conclusions regarding αvβ3 function drawn from these two sets of data. Compensation, a modification of protein expression during development to correct for the loss of an altered gene function, is raised as an issue where the phenotype of the transgenic animal does not meet expectations. On the other hand, the β3-specific reagents used in the inhibition studies were at one time poorly defined and the specificity may still be of issue but, if clarified, alternative conclusions may be drawn. In β3 knockouts, differences in results can not be explained by strain specific effects on angiogenesis as all animals exist in several backgrounds and several different tumor lines have been tested in the studies. Similarly, although highly unlikely based on high interspecies homologies, the conclusions in these studies have been drawn from different experimental species (genetics in mouse and interference studies in rabbit/ chick/ human) and could remotely underlie differences in the angiogenic pathways of these systems.

In a classic example where the functions of family members can overlap, the results of studies using α5 null fibroblasts determined that compensation had occurred

(Yang and Hynes, 1996). In this case, null fibroblasts did not upregulate surface expression of any other integrins but an increased recruitment of αv integrins into focal contacts was able to prevent adhesion defects in these cells. αv integrins were able to replace the function of the missing α5β1 integrin. The determination and assignment of function based on a phenotype of a targeted disruption can actually be quite complicated. Few proteins are targeted for ablation without an inclination that they play an important role. Investigators are occasionally surprised by an unexpected phentotype but not the lack of a phenotype. More generally, the lack of a noticeable phenotype often leads one to believe a compensation has occurred. In the case of beta 3 deficiency, the absence of a notable phenotype raised questions concerning the possibility of upregulation of other integrins to compensate for the loss of beta 3. Initial characterization of deficient embryonic fibroblasts from β3 deficient animals showed no changes in other integrin molecules (Hodivala-Dilke et al., 1999). Likewise, we have surface labeled MLEC and see no upregulation of expression of other family members (Reynolds et al., 2002).

Our results concerning VEGFR-2 and VEGF in $\beta3^{-/-}$ animals warrant discussion as to what is compensation. Genetic compensation extends to any changes in gene expression of any molecule which may correct for the absence of another gene product. For my discussion, I will not extend "genetic compensation" to include what may be a built in safety mechanism whereby there exists multiple layers of regulation and pathways controlling angiogenesis are so well intertwined that downstream effects of the loss of any one component changes the balance of activities and other existing angiogenic factors maintain a homeostasis. This kind of functional compensation could be as a result of changes in protein regulation inherent by the absence of a regulator. This regulation

may be direct or indirect (very indirect) through a signaling pathway which is no longer modulated due to the absence of beta 3 integrin. The two results of genetic ablation are certainly not mutually exclusive. It is unlikely that VEGFR-2 substitutes as an adhesion molecule for αvβ3 but the upregulation of expression may provide insight into the interaction between αvβ3 and VEGF signaling.

The functional relevance of the upregulation is not yet clear but it may be necessary for developmental angiogenesis in which αvβ3 plays a role distinct from that in pathological angiogenesis. The compensation likely contributes to the phenotype seen in pathological angiogenesis yet it is not the only contributor.

Additional mechanisms must also be considered in determining how the absence of beta 3 integrin fails to inhibit vascular development but actually enhances it. Many precedents for cross-talk, or transdominant inhibition, amongst integrins have been set and may play a role here. As discussed by Diaz-Gonzalez (Diaz-Gonzalez et al., 1996) transdominant inhibition initiated by integrin ligation may coordinate overall integrin signaling and lead to unexpected biological effects of integrin-specific inhibitors. The list of examples of transdominant inhibition by beta 3 integrins as well as other integrins is fairly extensive. β3 partnered with αIIb has the ability to inhibit both α5β1 and α2β1 (Diaz-Gonzalez et al., 1996). In cells expressing both α5β1 and αIIbβ3 but attached to FN solely through α5β1, the addition of an αIIbβ3 specific ligand suppressed α5β1 cell adhesion. The same was true for adhesion to collagen through α2β1. It is not clear how these effects were mediated but inhibition of inside–out signaling, and conformational changes in the extracellular regions of the affected integrin may play a role whereas cross-reactivity or interference by the αIIbβ3 specific ligand is not likely. Cross-talk

between integrins however is not always negative as is seen lymphocytes migration on VCAM-1 mediated by α4β1. This locomotion can be augmented by αvβ3 binding to VN or PECAM-1, likely dependent on αvβ3 associating with integrin associated protein (IAP) (Imhof et al., 1997). Similarly, in certain human tumor cell lines, addition of inhibitory antibodies for α3β1 was able to enhance cell adhesion to collagen through α2β1 (Lichtner et al., 1998).

Despite the lack of changes in integrin expression profile in β3-deficient animals, given the many examples sited above, αvβ3 may still be involved in the regulation of the function of other integrins in these cells. Specifically, the connection between αvβ3 and α5β1 in endothelial cell function has been made, albeit one in which αvβ3 is regulated by α5β1 (Kim et al., 2000b). Antibodies against α5β1 modulated migration on, but not adhesion to, vitronectin mediated by αvβ3 likely through the effects on small GTPases. The assumption that migration is a key aspect of angiogenesis extends these findings to suggest this cross-talk may occur in *in vivo* vessel development. Given the ability of αIIbβ3 to modulate α5β1 function, and α5β1 to regulate αvβ3 function it would be interesting if the αvβ3 integrin could regulate α5β1 in endothelial cells.

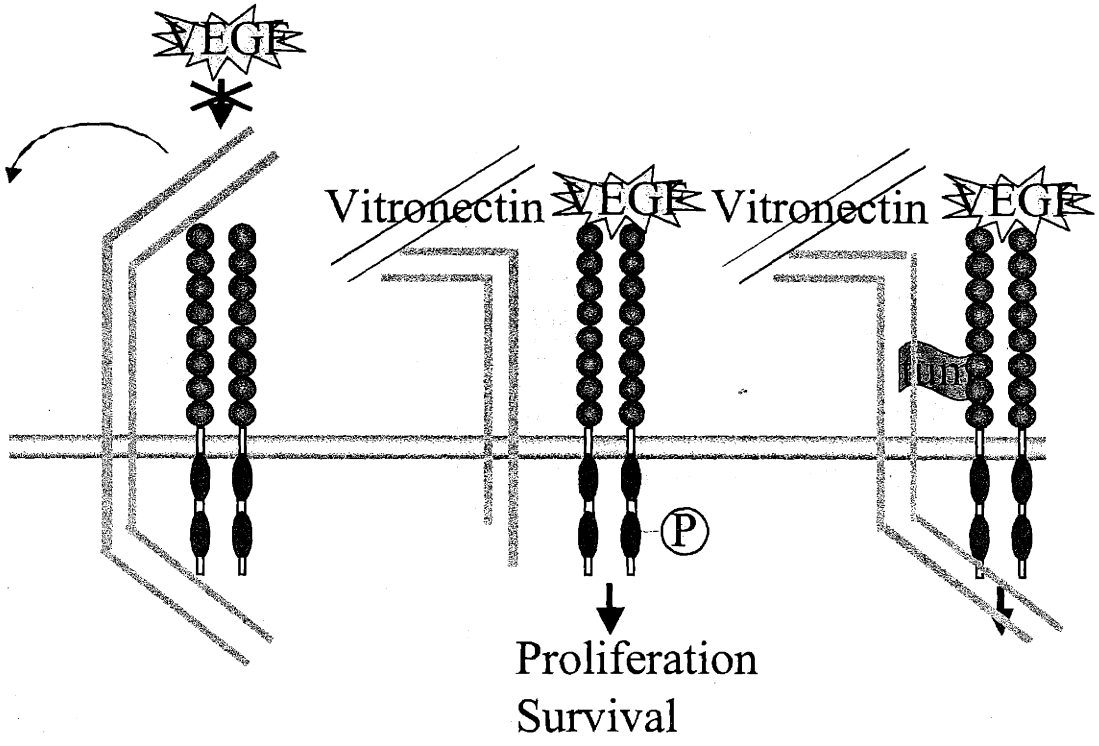
Although studied in a contrived cellular environment where αvβ3 was transfected into an α5β1 expressing erythroluekemia cell line, K562, antagonists of αvβ3 inhibited α5β1 mediated phagocytosis of FN-opsonized beads (Blystone et al., 1994), thus implicating that this regulation between integrins is plausible in either direction. Again adhesion is not inhibited in this case suggesting the disruption involves outside-in signaling of the targeted integrin by the dominant integrin.

The results of these αvβ3 integrin experiments certainly can support the possibility of trans-dominant inhibition in endothelial cells. The severity of the effect of alpha 5 deficiency on vascular development (Taverna and Hynes, 2001; Yang et al., 1993) suggests this integrin may be the most valuable in angiogenesis and it is possible that deficiencies of alpha v integrins release a transdominant inhibition of alpha 5 integrin thus promoting increased pathological angiogenesis through α5β1. In addition one could potentially incorporate "blocking" reagent experiments as well but must believe that these reagents activate integrin function. In addition to the cross-talk between family members, there is much evidence, albeit not enough to develop conclusions of mechanism or relevance, of cross-talk between integrins and growth factors.

Although transdominant inhibition typical refers to the regulation of one integrin by a second integrin, there is no reason why evidence such as that discussed in Chapter 3 involving αvβ3 and VEGFR-2 could not be another example of this principle. The data suggest that unliganded αvβ3 is capable of inhibiting VEGFR signaling. To extend the possible interplay of αvβ3 binding to either VEGFR-2 or VN, Soldi introduced antibodies which did not interfere with VN binding but did reduce tyrosine phosphorylation of VEGFR-2 and the biological function of VEGF engagement (Soldi et al., 1999). This particular reagent may actually fall into a class including tumstatin which function to maintain the β3: VEGFR interaction and inhibit signaling through VEGFR (See Figure 5.1).

Figure 5.1: Modified model of tumstatin: αvβ3:VEGFR-2 interaction

To extend the model from Chapter 3, I am hypothesizing that one function of tumstatin, and reagents like this, is to stabilize the interaction between these two receptors and thus act to enhance the antiangiogenic role of αvβ3 integrin.



Friedlander's model which suggests a relationship between angiogenic growth factors, bFGF and VEGF, and endothelial integrins, αvβ3 and αvβ5, respectively, is based on the assumption that the blocking reagents used in his experiments inhibit the function of a positive regulator of angiogenesis (discussed in Chapter 3.1). As our recent data suggest (Reynolds et al., 2002), beta 3 inhibits VEGF-induced angiogenesis *in vivo* and blocking this function would permit an increase in VEGF-induced angiogenesis. In fact, β3-deficient endothelial cells are more responsive to VEGF *in vitro* further supporting this model. To examine exhaustively the correlation between growth factor-

induced angiogenesis and integrin function, one would also need to examine bFGF-induced angiogenesis in beta 5-deficient animals. If we assume β5 functions similarly, we would reinterpret the results to suggest β5 integrin regulates bFGF-driven proliferation and angiogenesis. It is likely that Friedlander's blocking reagents in fact stimulate the inhibitory roles of αv integrins in the vasculature. To further understand the roles of αvβ3 and αvβ5 in growth factor signaling, more investigation is necessary. Matters are complicated by the regulation of VEGFR by bFGF. Treatment of cells with bFGF results in the upregulation of proteins kinase C and MAP kinase ultimately leading to the upregulation of VEGFR-2 (Hata et al., 1999). In a system with exogenous bFGF added, results may be complicated by unintentional signaling through VEGFR. Is the VEGF signal predominant and could the results of Friedlander actually be the result of two independent events which both impinge on the VEGF signaling pathway? That is, the engagement of beta 3 by antibody activates it and downregulates VEGFR-2, the addition of bFGF to this system is not actually stimulating an independent pathway but simply feeding into the VEGF pathway which is already blocked. Essentially a portion of bFGF-induced angiogenesis can be attributed to VEGF and is therefore inhibited by αvβ3 "antagonists" and not αvβ5 "antagonists". In light of the possibility that all aspects of these two pathways are reciprocal, more data is needed to understand the αvβ5 "antagonist" experiments as well.

Other genetically modified cells have been generated which could also be used to test cellular responses to VEGF and bFGF. To clarify the results of Friedlander, et al. (1995) one could examine the effects of bFGF treatment in VEGFR-2 deficient cells. If the angiogenic stimulus which is abrogated by αvβ3 antagonists is strictly due to a bFGF

pathway and not the subsequent upregulation of the VEGF pathway one would expect the same order of magnitude of inhibition in VEGFR-2-/- cells.

Additional studies can also support an inhibitory role for αvβ3. Brooks et al (1994b), have reported that injection of cyclic RGD or "antagonistic" antibodies to αvβ3 induce endothelial apoptosis and they conclude that αvβ3 integrin is essential for the survival and maturation of vessels. We propose that the formal possibility exists that this result may provide additional information of αvβ3 integrin as a negative regulator of angiogenesis. In fact, no obvious increases in apoptosis were found in beta 3-null vessels. These inteference reagents are engaging αvβ3 and in the case of antibodies clustering this integrin and may be stimulating it's natural antiangiogenic role. Binding to VEGFR-2 would be enhanced and survival signals through this receptor tyrosine kinase would be abolished. Again given the delicate nature of vessel integrity, the regulation of VEGFR is unlikely the only role of β3 integrin.

Additional to any antiangiogenic activity suggested above, evidence that I present herein also shows that ligation of beta 3 integrin with tumstatin leads to protein synthesis inhibition. The identification and characterization of a novel function for beta 3 integrin as a receptor for an endogenous inhibitor of angiogenesis additionally defines its role not as a pro-angiogenic molecule but as an antiangiogenic molecule which correlates nicely with the lack of an angiogenic defect in beta 3-deficient animals. Perhaps beta 3 upregulation on stimulated or "active" endothelium exists in preparation of the need to attenuate the process in an effort to avoid excessive and potentially harmful angiogenesis.

These studies with tumstatin have contributed to the understanding of the role that beta 3 integrin may play *in vivo*. Prior to the generation of a grossly normal αvβ3

knockout animal, predictions were made that pathological angiogenesis would follow a slightly different paradigm and would be impaired in these animals. Studies with beta 3-deficient adult animals, as well as cells isolated from them, have continued to yield results which do not conform with previous published work of beta 3 inhibitors which suggested a pro-angiogenic role for this molecule. Models have yet to show any vasculogenic deficiency, and in fact, an increase in tumor size is seen and subsequent analysis suggests an increase in vascular density. Several non-mutually exclusive theories are presented to explain this phenotype, many of which data in this thesis support.

Beta 3 integrin has a role binding to endogenously produced tumstatin, a cleavage product of vascular basement membrane, and downregulating angiogenesis. To formulate theories of mechanism, it is important to think of the sequential timing of events in angiogenesis. Proangiogenic stimuli, including MMP production, VEGF release and activity, and integrin engagement, induce proliferation, migration and formation of new vessels. MMPs also may produce tumstatin along with other matrix degradation products which are endogenous inhibitors of angiogenesis. From this point it becomes a tug-of-war. Are there more pro-angiogenic or anti-angiogenic signals? Have matrix degradation products reached a critical concentration at the site of angiogenesis? Tumstatin can bind to and activate a cascade of inhibitory signals through the upregulated alpha v beta 3 integrin molecule on the surface of endothelium. Although I would like to convince you the antiangiogenic activity of tumstatin through beta 3 is in fact the normal function of this receptor, there is no absolutely clear data which refutes the theory that

tumstatin is acting as an antagonist to block normal pro-angiogenic signals through β3 integrin.

Tumstatin's ability to inhibit protein synthesis so far appears restricted to endothelial cells. As discussed in Chapter 4, the VEGF signaling cascade is an interesting possibility which may impart endothelial specificity to tumstatin function. A synergy of signals between GF action and integrin action on a cell is an attractive model of tumstatin activity, temporally as well as spatially, in angiogenesis.

The identification of an activity which inhibits angiogenesis through αvβ3 also is a contributing factor in the analysis of the pathological phenotype of β3-null mice. The production of this inhibitor, and potentially others including TSP-1 and endostatin, which may jointly inhibit angiogenesis through β3 may have been underrepresented in interference studies and therefore not considered in the analysis of β3 function. These inhibitors would be additional factors that could resolve the discrepancies between the two theories of beta 3 function and tilting the balance towards the importance of beta 3 as a negative regulator of angiogenesis.

In a somewhat different line of thought, a noticeable upregulation of VEGF and VEGFR reminds me of the end results of endothelial cell hypoxia. Interestingly hypoxia, while negatively regulating many genes, allows for CAP-independent synthesis of VEGF (Stein et al., 1998). Endogenous production of tumstatin, along with other similar matrix degradation products, in tumors inhibits CAP-dependent protein synthesis yet it is likely that hypoxia-inducible factors such as VEGF are still promoting proliferation and formation of blood vessels. Related to hypoxia, tumstatin engagement activates only caspase 3 and not caspase 8. Reports show that caspase 8 is not involved in hypoxia-

mediated cell death whereas caspase 3 is upregulated (Tezel and Wax, 1999). Interestingly if β3 is playing a role in the hypoxia-inducible pathway one might expect such differentiation between caspase upregulation. If β3 plays a role in inhibiting the hypoxia response, it is also not surprising that in the absence of β3, VEGF and VEGFR are upregulated and increased angiogenesis is observed. Although the data don't appear complete, Kroon et al (2000) showed that LM609, an αvβ3 specific reagent, was unable to influence hypoxia-induced capillary formation of hMVECs (human microvasculature) on a 3-dimensional fibrin clot. In support of αvβ3 as an inhibitor of hypoxia-induced angiogenesis, and my working hypothesis that LM609 is in fact an agonist of αvβ3, one would expect inhibition of angiogenesis, however hypoxia also upregulated expression of αvβ3 integrin and this may be sufficient to counter the effect (Kroon et al., 2000). Consistent with this, retinopathy of prematurity (ROP) studies in beta 3-deficient mice also showed an increase in vasculaturePutting together the *in vitro* analysis of beta 3 -/- MLEC function as well as tumstatin effects through beta 3 integrin, a new theory is emerging which can help explain the delicate balance that is achieved between normal and pathological angiogenesis in the adult. As important as it is to the survival of tumors and to successfully repair wounds, initiation of the angiogenic process may be balanced with the equally important fact that there is a series of events which attenuates and terminates this pro-angiogenic process.

The regulation of the development of new vasculature is achieved by the dual pro-angiogenic and anti-angiogenic properties of critical components. Matrix metalloproteinases are required for the degradation of such molecules as collagen to stimulate migration of endothelial cells and conversely the degradation products attenuate

angiogenesis by inhibiting protein synthesis and proliferation of endothelial cells. The expression of beta 3 certainly does not inhibit angiogenesis a priori and may, as previously concluded, have a pro-angiogenic role at some point in development, and thus another example of an angiogenic molecule both promoting and inhibiting angiogenesis.

Until further experimentation is completed, I would like to devise a hypothetical scenario based on the data provided. αvβ3 integrins function in normal endothelium to inhibit or modulate angiogenesis not stimulate it as originally predicted. Would-be antagonists of αvβ3 could actually be functioning to activate αvβ3 function. In the absence of αvβ3, angiogenesis not only occurs but is enhanced in pathological conditions. These "antagonists" interact with αvβ3 in its ligand-binding domain and may act as ligand mimetics. In the case of tumstatin, which interacts at a site distinct from and non-interfering with the ligand-binding site, it is unclear what events are activated downstream and if these are identical to the antiangiogenic stimulus of previously identified reagents.

Chapter 6: Methods

1. Isolation and Culture of Primary Mouse Lung Endothelial Cells

Lung endothelium was isolated from 6-10 week-old mice essentially as concurrently reported by Dong et al. (1997). Dong reported a method utilizing PECAM-1 antibody-coated magnetic beads to purify an endothelial population from a heterogeneous lung cell population. Amounts in this method represent what is typical for 1-2 mice. Briefly, mice were sacrificed by cervical dislocation. The thoracic cavity was opened above the diaphragm exposing the heart and lungs. Perfusion of the lungs with approximately 8ml heparin (1u/ml) in PBS was accomplished with assistance by the still-beating heart by injecting into the right ventricle and snipping the right atrium. The heart and lung were removed and placed in isotonic medium (Ham's F-12, Gibco BRL). In a sterile environment, each lobe was dissected from the bronchial tube, dipped in 70% ethanol to remove the mesothelium, and transferred to MLEC medium. After the dissection of all lobes, they were transferred to a dry sterile tissue culture dish lid and minced. Minced tissue was digested 1hour at 37°C in 1% collagenase (pre-treated at 37° for 1 hour, Worthington) in PBS, with occasional agitation. Suspensions were passed through a sterile, 140-μm mesh screen (Bellco Glass Inc. Vineland, N.J.) to remove undigested tissue. MLEC medium (1-2 equivalents) was added and dissociated cells spun at 329 x g (1200rpm), 5 min., 4 °C to collect cell pellet (Beckman, model GS-6KR). MLEC were seeded onto one 75mm^2 tissue culture plate coated with 30 μg/ml denatured rat tail collagen (Vitrogen, Collagen Corp., Palo Alto, CA), 10μg/ml rat FN (Sigma or Gibco BRL) and 0.1% gelatin (Sigma) at a density of ~2-4 x 10^5 cells/cm^2. Cultures were grown in MLEC base medium consisting of 40% Dulbecco's Modified Eagle's- Low Glucose Medium (DME-LG; BioWhittaker, Walkersville, MD, USA), 40% Ham's F12 (BioWhittaker, Walkersville, MD, USA) supplemented with 20% fetal bovine serum (FBS)

(Hyclone,USA). Growth medium was supplemented with 100 μg/ml heparin (Sigma), 50 μg/ml Endothelial Mitogen (Biomedical Inc., Stoughton, MA, USA), 200mM L-glutamine (Gibco BRL), 100 i.u./ml penicillin and 100 μg/ml streptomycin (Gibco BRL). Antibody-coated magnetic beads were prepared for both negative (FcBlock) and positive (ICAM-2) cell selections. Dynabeads® M-450 Sheep anti-Rat IgG (Product No. 110.07 or 110.08, Dynal, Norway) chemically conjugated to anti-rat IgG (50μl) were incubated with antibody (10μl of 0.5mg/ml) in 500 μl of 2% FBS in PBS at 4°C at least overnight. Antibody coated beads could be stored for 6 months at 4°C. On day 1 in culture, well washed flasks containing only adherent cells were chilled at 4°C for 10-30 minutes to reduce membrane fluidity (phagocytosis and endocytosis). Beads coated with FcBlock (Pharmingen) were washed to remove excess unconjugated antibody and resuspended in 3ml cold MLEC medium. Beads were incubated with cells for 1hour at 4°C with occasional shaking to redistribute beads. Unbound beads were removed (and saved) and cells were washed well and trypsinsized. Cells expressing FcγRII, primarily macrophages, bound beads and were depleted using Dynal MPC (magnetic particle concentrator). Cells not bound to the magnet were replated into one T75 coated flask. On day 3, beads coated with ICAM-2 (Pharmingen, Clone 3C4 (mIC2/4)) were used to enrich by the same method yet harvesting cells conjugated to beads and drawn to the magnet. ICAM-2-positive cells were plated onto one T25 coated flask. A final positive selection was repeated when the flask reached 80% confluent and positive cells were plated onto one T75 coated flask. Mouse lung endothelial cells (MLEC) were cultured at 37°C, 5% CO2 for up to passage 6 in MLEC medium (described above).

2. Establishment of conditionally immortalized MLEC lines

To generate conditionally immortalized cell lines from wild-type and β3-deficient endothelium, we exploited the ImmortoMouse (Charles River Laboratories, Wilmington, MA, USA), which expresses a temperature-sensitive mutant of SV40 large T antigen (tsA58) under the control of the interferon γ (IFNγ)-inducible H-2K^b promoter (Jat et al., 1991). Mice null for β3-integrin (Hodivala-Dilke et al., 1999) were bred with the Immortomouse to generate β3 +/– mice that contained the H-2K^b- tsA58 transgene (Im $^+$). These mice were then mated and offspring genotyped by PCR for the β3-null mutation as described previously (Hodivala-Dilke et al., 1999) and the presence of at least one copy of the H–2K^b–tsA58 transgene was confirmed by PCR using a forward primer (AGCGCTTGTGTCGCCATT GTATTC) and a reverse primer (GTAACACCACAGAAGTAAGGTTCC) that produced a PCR product of approximately 1000 base pairs in the following PCR reaction: 95°C, 1 minute; 58°C, 2 minutes; 70°C, 3 minutes; 38 cycles.

Primary endothelial cells were isolated from the β3 -/- Im + offspring essentially as described above. Due to minimal health issues arising from the presence of SV40 LgT, primary endothelial cells were prepared from slightly younger mice, 6-8 week-old mice. Endothelial growth medium consisted of 40% Dulbecco's Minimum Essential Medium- Low Glucose (DME-LG; BioWhittaker, Walkersville, MD, USA), 40% Ham's F12 (BioWhittaker, Walkersville, MD, USA) supplemented with 20% fetal bovine serum (FBS) (Hyclone, USA). Growth medium was supplemented with 100 μg/ml heparin (Sigma), 50 μg/ml Endothelial Mitogen (Biomedical Inc., Stoughton, MA, USA), 200mM L-glutamine (Gibco BRL), 100 i.u./ml penicillin and 100 μg/ml streptomycin (Gibco BRL). Cell cultures were expanded approximately 10-12 passages. Cells were cultured at permissive temperatures (33°) in growth medium supplemented with 50

units/ml IFNγ (RD, Cambridge, MA, USA, or Gibco BRL) for maintenance or shifted to semi-permissive conditions (37°) in the absence of IFN-γ for assays. Cells examined at 37° show typical endothelial morphology and express specific markers. Antibody detection of active SV40 large T antigen was minimal at 37°C and high at 33°C.

Antibodies

Antibodies were used at designated dilutions as outlined below. Manufacturer information and clone information for all antibodies used are listed as well.

Antigen	Raised in:	IF dil^n	FACS dil^n	Manufacturer	Clone #
α-catenin	rabbit	1/500		Sigma	
β-catenin	rabbit	1/500		Sigma	
flk-1	rabbit	1/100		Santa Cruz	sc315
ICAM-1	rat	1/3	1/2	supernatant	J.C. G.-Ramos
ICAM-2	rat	1/100	1/100	Pharmingen	3C4(mI2/4)
integrin α5β1	rat	1/25		supernatant	MFR5
integrin β3	hamstr/bio conj.	1/50		Pharmingen	
PECAM	rat	1/100	1/100	Pharmingen	MEC13.3
Selectin, E-	rat	1/100	1/100	Pharmingen	10E9.6
Selectin, P-	rat	1/100	1/100	Pharmingen	RB40.34
sv40	mouse	1/100		Oncogene Science	
Tie-1	rabbit	1/100	1/100	Santa Cruz	sc342
Tie-2	rabbit	1/100	1/100	Santa Cruz	sc324
VCAM-1	rat	1/20	1/20	supernatant	yellow tubes
VE-Cadherin	rat	1/50	1/2	Pharmingen	11D4.1
vWF	rabbit	1/100		Diagnostica Stago	

3. Immunofluorescence staining

Endothelial cells were fixed in 3.7% formaldehyde in PBS, blocked with 10% Normal Goat Serum (NGS, Sigma) in PBS and permeabilized with 0.5 % NP-40/PBS. Primary antibodies diluted in 10% NGS as outlined above for 1 hour. Appropriate secondary antibodies, either conjugated to a fluorophore or to biotin followed by amplification with an avidin-fluorophore

incubation were used from the AlexaFluor series (Molecular Probes), For β3 biotinylated primary antibody avidin-FITC was used.

4. FACS analysis

All dilutions and rinsing were done using 1% BSA in PBS unless otherwise noted. Adherent MLEC cultures were trypsinized (EDTA detachment was not efficient) and rinsed. Single cell suspensions of wild-type and β3-null endothelial cells were incubated with antibody on ice. Cells were washed and incubated with a FITC-conjugated secondary antibody. The cells were then washed and resuspended in FACS buffer (PBS, 2 % FCS, 0.02 % NaN_3) and analyzed for fluorescence using a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). For negative controls the primary antibody was omitted.

5. Tube Formation Assays:

a. Spontaneous

MLEC were plated onto gelatin/ vitrogen/ fibronectin-coated dishes (as described in MLEC culture) at $1x10^3$ cells/mm2 and incubated for 24-72 hours in full 20% FBS-containing medium. Photographs were taken with a Kodak MDS 290 digital microscope accessory kit on a Zeiss microscope.

b. Microcarrier Bead Assay

A very good manual is available from Pharmacia Biotech describing the Microcarrier cell culture: principles and methods (# 18-1020-00). Cytodex Microcarrier 3 beads (Cytodex-3, Pharmacia Biotech) were coated with gelatin, vitrogen, and fibronectin (as described in MLEC

culture) and counted. Cells were attached to beads approximately 100 cells/bead (500 μl beads at 10 beads/μl) for 4 hours. The first 30 min without shaking and then minimal shaking every 20 minutes for the duration. Once cells are attached to beads, they were cultured at 37°C for 2-4 days in suspension. Only occasional shaking of the flask was necessary to minimize adhesion to surface or clumping. Beads coated with cells were then imbedded in fibrin (fibrinogen plus thrombin) and observed for vascular sprouting.

6. Ex vivo Aortic Ring Angiogenesis Assay

Using a similar dissection method as described above for mouse endothelial cells, mouse aortae were dissected and separated from the heart and lungs. Under the dissecting microscope, fat was removed from the outer walls of the aorta. 1.5mm-2mm cross-sectional pieces of aortae were laid on 30 μl pre-solidified Matrigel (BD Biosciences, Bedford MA) in a 48-well cluster dish. An additional 30μl Matrigel was overlaid on the aorta and allowed to solidify at 37°C. Serum free- or low serum- MLEC medium (50% Dulbecco's Modified Eagle's- Low Glucose Medium (DME-LG; BioWhittaker, Walkersville, MD, USA), 50% Ham's F12 (BioWhittaker, Walkersville, MD, USA) 100 μg/ml heparin (Sigma), 200mM L-glutamine (Gibco BRL), 100 i.u./ml penicillin and 100 μg/ml streptomycin (Gibco BRL), 0 or 2% fetal bovine serum (FBS) plus experimental serum/growth factors additives) was added to embedded aorta. Aortic rings were monitored daily for 10 days, photographed using the Kodak MDS 290 Digital Camera System and sprouts were counted. For graphical representation, sprouts/aortic ring were adjusted using the following calculation: (Total number of sprouts/unit length of aorta) * average aortic ring diameter. Measurements of aortic ring diameter were made from photographs of ring

images at identical magnifications. Aortic rings were very consistent in size (average 34.8 Units +/- 1.7 (n=40))

7. Matrigel® Plug Assay

Five to six week-old male C57/BL6 mice were preferred but male and female mice up to 15 weeks have been used in this assay. Matrigel® (BD Biosciences, Bedford MA) was thawed overnight on ice at 4°C to ensure constant temperature. Mice were anesthetized using ≈ 0.3ml of a 1/40 dilution of avertin stock solution (2,2,2-tribromoethanol in tert-amyl-alcohol [25g/26ml]) diluted in PBS. All pipettes, Eppendorfs, syringes and needles were cooled overnight at 4°C. 20U/μl heparin (Sigma) diluted in water was filter-sterilized. Matrigel® mixture was prepared on ice at 4°C containing 20U/ml heparin, growth factor and treatment. VEGF (R & D Systems) was used at 50ng/ml unless otherwise noted. bFGF (Scios Nova) was used at 250ng/ml unless otherwise noted. A cold 1ml syringe (sans needle) was used to slowly mix the solution. Controls included Matrigel® and heparin or Matrigel®, heparin and growth factor. 21 gauge needles were attached to the syringes and were kept on the ice as long as possible before injection. Animals received subcutaneous injections of 200, 300 or 450μl of the Matrigel® mixture in a dorso-lateral position. Each mouse was injected on both sides. Mice were monitored for 7 days at which time they were sacrificed by CO_2 asphyxiation and the Matrigel® plugs removed. The plugs were surgically excised and fixed in 3.7% formaldehyde overnight. The excision was done in such a way that the plugs remained attached to either the peritoneum or the skin and remained attached during an initial fixation (minimum 3 hours) the Matrigel plugs were then detached by scraping with a razor blade and stored in cassettes in 70% EtOH prior to sectioning by Denise Crowley (histologist extraordinaire).

8. Adhesion assay

96-well bacteriological plates (Nunc, Rochester, New York) were coated with 10 μg/ml of fibronectin, vitronectin, collagen or laminin in PBS overnight at 4°C. The plates were washed briefly and then blocked in 10 mg/ml BSA in PBS for 2 h at 37°C. Cells (5 x 10^4) in 100 μl of serum-free medium containing 25 μM cycloheximide were plated per well for 1 hour at 37°C. Adherent cells were fixed and stained in 1% crystal violet in H_2O and counted.

9. Proliferation assay: Methylene Blue Staining

Contact-inhibited MLEC growing in normal medium (48 hours) were trypsinized and resuspended at 2.5 x 10^4 cells/ml in base medium containing 0.1% FBS. 100μl of cell suspension was added to each well of a 96-well plate precoated with matrix. Cells were serum starved for 24 hours at 37°C , 5% CO_2. Medium was changed to 20% FBS MLEC medium (normal medium) containing varying treatments. Cells were incubated 24 hours 37°C, 5% CO_2. Samples were fixed with 3.7% formaldehyde, 10 min., rinsed and stained with methylene blue for 0.5 hours. Samples were rinsed well and methylene blue is extracted for 20 min. Extracted vital stain was quantified at 655nm on a Molecular Devices plate reader.

10. Proliferation assay: [^{3}H]-thymidine incorporation

Contact-inhibited MLEC growing in normal medium (48 hours) were trypsinized and resuspended at 2.5 x 10^4 cells/ml in base medium containing 0.1% FBS. 500μl of cell suspension were plated into each well of a 24-well plate pre-coated with 10 μg/ml fibronectin

(200 mL/well). Cells were serum-starved for 24 hours at 37°C with 5% CO_2 and 95% humidity. Cells were shifted into complete medium (containing 20% FBS) containing varying concentrations of treatment (see text) for 12 hrs at 37°C. Unstimulated control cells were incubated with medium containing 0.1% FBS. Twelve hours after beginning treatment, cells were incubated for 24 hours with 1 μCi/well of [^{3}H]-thymidine. Rinsed cells were incubated for 30 min at 37°C with 100 μL of 1N NaOH per well. Thymidine incorporation was measured in a scintillation vial containing 4 ml of ScintiVerse II (Fisher) solution using a scintillation counter.

11. BrdU (bromodeoxyuridine) Incorporation

BrdU proliferation assay kit (Calbiochem) was followed according to the manufacturer's instruction with some modifications. MLEC were seeded onto coated 96-well plates (10^4 cells /ml = 31 x10 2 cells per well) in medium containing 20% Fetal Bovine Serum. The following day the medium was replaced with reduced serum medium (2% FBS) with or without peptide treatment. The cells were cultured for 46 hours, 37°C, 5% CO^2 and then pulsed for 2 hours with BrdU (10nM) Samples were fixed to the wells, reacted with anti-BrdU primary and secondary antibodies and then developed using a colorimetric reaction. The plates were read at OD 450nm on a Molecular Devices plate reader. All groups represent triplicate samples.

12. Protein synthesis.

Cells were serum-starved for 24 hrs (0.5% FCS), stimulated with 20% FCS in the presence of tum-5, T3 peptide, T7 peptide,T7 alpha 5 mutant peptide, endostatin or rapamycin for 12-24 hours. Cells were incubated 1 hour in methionine-free medium. Cells were then labeled with

[^{35}S]-methionine-containing medium for 1 hr and incorporation of radioactivity in trichloracetic acid precipitates was analyzed.

13. Transfection of plasmid DNA into MLEC

Lipofectamine transfection protocol was followed according to manufacturers instructions with slight modifications. In summary, MLECs were plated (0.08-0.1 million cells/1 well of 6-well plates) on coated plates (gelatin, FN, Vitrogen) in complete MLEC medium containing 20% FBS for more than 12 hrs. Cells were then serum- deprived (incubated in MLEC medium lacking endothelial mitogen (BTI) and containing only 5% serum) for 24 hours. One half-hour prior to transfection, DNA and 100 μL of medium per 1 well were mixed. Before use "Plus reagent" mixed and added to DNA, mixed, and incubated at RT for 15 min. In the last 5 min, the "LIPOFECTAMINE Reagent" was diluted with medium. 4 μL of LIPOFECTAMINE Reagent and 100 μL of medium per 1 well was used. The DNA mixture and Lipofectamine mixture were mixed and incubated for 15 min at RT. Cells were incubated for 3 hrs in serum-free medium (800μl) and DNA-PLUS-Lipofectamine Reagent(211μl). Medium was replaced with complete medium with 20% serum. Resultant transfected MLEC population was used for treatment experiments. Treatment added, and incubated for 24-72 hrs (48hr-72hr is better). Experiment was repeated in triplicate.

14. CAP-dependent translation analysis Cells transiently transfected as above with pcDNA3-LUC-pol-CAT (Kumar et al., 2000) and pRL-TK (Promega) using Lipofectamine Plus (Life Technologies) were used to assay CAP-dependent protein synthesis (Maeshima et al., 1998). Measurement of firefly luciferase activity correlates to CAP-dependent translation in transiently

transfected cells and was measured according to manufacturer's instructions for the Dual Luciferase Reporter Assay System from Promega with slight modifications. In summary, transfected MLEC were washed with PBS and lysed 15 min at room temperature with gentle rocking in 1x kit lysis buffer (200 μL per well; Luciferase Assay kit or CAT assay kit from Promega). Cells were scraped off dishes and lysates transferred into microcentrifuge tubes. Cell lysates were kept still for 10 min at room temperature. Lysates were centrifuged 5 min. at 13,000 rpm at room temperature. 20 μL of cell lysate were transferred to 4ml polypropylene FACS tube. 100 μL of "B" substrate solution was added to lysate and immediumtely put into luminometer without vortexing or pipetting. Sample tube was removed and 100 μL of "S" (STOP and Glo) substrate solution was added. Sample was vortexed for 4 seconds and luminescence measured in a luminometer.

After the measurement, the rest of the supernatant was transferred into a new microfuge tube and kept at -20°C. Firefly luciferase activity divided by Renilla luciferase activity corrects for the transfection efficiency.

15. CAT assay

Chloramphenicol acetyltransferase activity was measured using CAT assay kit from Promega according to manufacturer's instructions. In summary, transfected MLEC were washed with PBS and lysed 15 min at room temperature with gentle rocking in 1x kit lysis buffer (200 μL per well; CAT assay kit from Promega). Cells were scraped off dishes and lysate transferred into microcentrifuge tubes and kept on ice. Half of cell lysate was vortexed for 10 seconds, incubated at 60°C for 10 min, centrifuged 3 min. at 13,000rpm at 4°C in microcentrifuge and supernatant collected to new microcentrifuge tubes. Cell lysates were stored at -80°C.

Reaction mixtures prepared with cell lysate, 0.15 mCi [^{14}C]chloramphenicol, n-Butyryl CoA. CAT (1U/reaction), diluted in equal volume of lysis buffer as in experimental samples was used as positive control. Lysis buffer alone was used as negative control. Reactions were incubated at 37°C for 3 hrs. Touch spin down reaction mixture. 300 μL of mixed xylenes were added to each reaction. Samples were vortexed for 30 sec. and centrifuged 3 min. at 13,000rpm and the upper phase (xylenes) transferred to a fresh tube. Equal reaction volume maintained. 100 μL of 0.25M Tris-HCl (pH8.0) was added, vortexed for 30 sec and centrifuged for 3 min. at 13,000rpm. Radioactivity in the upper xylene phase measured in a liquid scintillation counter. Counts measured in negative control (no enzyme) were subtracted from each sample to determine the specific level of n-butyrylated products.

16. Western blotting

Cell lysates were prepared from primary MLEC or Im+ MLEC in RIPA buffer (1% Triton-X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 10 mM Tris, pH 7.3, 1 mM EGTA, 2 mM PMSF, 12.5 μg/ml leupeptin, 15 μg/ml aprotinin) and quantitated using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Following cell extractions, ECM fractions were scraped from plates into reducing SDS-sample buffer (2% SDS, 80 mM Tris, pH 6.8, 2 mM EDTA, 100 mM dithiothreitol, 2 mM PMSF, 12.5 μg/ml leupeptin, 15 μg/ml aprotinin). Protein preparations were subject to nonreducing 10% SDS/PAGE for β3 integrin blots (5 μg/lane) or reducing 7% SDS/PAGE for VE-cadherin blots (25 μg/lane), then transferred to 0.2 μm PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blotted with primary antisera at the following dilutions: anti-VE-cadherin 14, 1:200; anti-catenin (α, β or γ subunit), 1:1000. Peroxidase (HRP)-conjugated goat anti-rabbit IgG (Pierce) was used as secondary antibody at 1:15,000 dilution. Chemiluminescence was performed with the SuperSignal Kit (Pierce). For western

blotting of MMP-9, gelatinases were concentrated from culture medium by binding to gelatin-agarose beads (Sigma). Medium from Im + MLEC cultures (2 ml) or primary endothelial cells (1 ml) was incubated overnight at 4°C with 100 µl of gelatin-agarose. Control incubations included purified murine MMP-9 or human MMP-2/MMP-9 mix in DME-LG/Ham's. Agarose beads were recovered by centrifugation and eluted in electrophoresis sample buffer. Samples were immunoblotted with goat polyclonal antiserum against MMP-9 (1:200 dilution) followed by HRP-conjugated anti-goat IgG (Santa Cruz Biotechnology) at 0.4 µg/ml.

17. Gelatin zymography of MLEC culture medium

Equal numbers of MLEC were attached to GVF overnight, then rinsed and fed with serum-free medium that was otherwise fully supplemented, as described above. Cells were cultured for 48 hours (37°C). Culture medium was collected for zymography, and lysates were prepared from the cell monolayer for western blots (see above). For some experiments MMPs were concentrated from culture medium by binding to gelatin-agarose beads (see below). Equal volumes of culture medium were mixed with zymography sample buffer (final: 2.25% SDS, 9% glycerol, 45 mM Tris, pH 6.8, Bromophenol Blue) and resolved by nonreducing SDS/PAGE on 10% polyacrylamide gels impregnated with 1 mg/ml gelatin (Sigma). Following electrophoresis, gels were soaked in 2.5% Triton-X-100 to replace SDS, washed twice with water, then incubated at 37°C for 10-24 hours in MMP activation buffer (50 mM Tris, pH 8.0, 5 mM $CaCl_2$). In control experiments, calcium was replaced with 10 mM EDTA in the activation buffer. For inhibitor studies, 5 mM PMSF (Sigma; isopropanol as solvent) or 10 mM 1,10-phenanthroline (Sigma; methanol as solvent), or solvent only, was added to the Triton and activation buffers. For activation studies, samples were preincubated with 1.5 mM p-aminophenylmercuric acetate

(APMA; Sigma), or solvent only (DMSO), at 37°C for 6 hours prior to electrophoresis. Zymography gels were stained with Coomassie Blue and destained in 10% methanol, 5% acetic acid. Proteins with gelatinolytic activity were revealed as clear bands on a blue background. Purified murine MMP-9, or a mixture of human MMP-9 and MMP-2 (Chemicon International), served as a positive control.

18. Corneal Micropocket Angiogenesis Assay

Slow release pellets containing growth factor were prepared basically as described in Kenyon (1996). A slurry of growth factor in solution (≈20 μl, 20 μg rbFGF or 50 μg rVEGF) and sucralfate (10mg, Sigma) was made and dehydrated (≈10min in rotovac). Hydron polymer (60mg, Type NCC, Interferon Sciences) in ethanol (500μl) vortexed 10 min. Sucralfate/growth factor resuspended in hydron solution, mixed with sterile spatula and quickly spread on sterile nylon mesh. Consistency was checked by holding up to a light, more dense spots were spread more. A thin layer of hydron solution was spread on both sides of mesh to seal pellets. Pellets were cured 30 min at RT. Mesh was pulled apart thread-by-thread in bacteriological 10cm plate to release pellets which were then stored at –80°C. Topical anesthetic was applied to the eye of an animal under general avertin anesthetic. A temporal incision partially through the cornea was made with a 15 degree knife (Surgistar). A triangular pocket was made with a microdissection knife perpendicular to this incision approximately 1mm from the limbic vessel. The pellet was inserted into this pocket and antibiotic ointment applied. After 7 days, eyes were analyzed for neovascular growth.

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