Vascular Morphogenesis in the Female Reproductive System

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CHAPTER 5

Oncogenes, Anti-Oncogenes, and Genetic Regulators of Vascular Development

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The architecture of all tissue represents the cumulative actions of many genes. Similarly, the phenotype of tumors is the product of successive genetic lesions that alter the function and regulation of oncogenes, tumor-suppressors, and modifier genes. A key stage of both tissue development and tumor progression is the recruitment of a vascular network.

From limited experimental and epidemiologic data, it is hypothesized that the response of tumors to specific therapeutic agents may vary considerably (Thompson et al, 1999). In particular, various tumor cell lines produce diverse levels of angiogenic growth factors and respond differently to antiangiogenic therapies (Kim et al, 1993; Takahashi et al, 1996; Yuan et al, 1996). It is therefore critical to understand the contributions of tissue-specific and genetic factors to the vascular response. It is likely that variability in vascular responsiveness will complicate the search for global modulators of angiogenesis. Conversely, such variation may ultimately provide a benefit by increasing the potential for target specificity in distinct disease states.

While there is solid evidence that the progression of solid tumors involves angiogenesis (reviewed in D'Amore and Shima, 1996; Hanahan and Folkman, 1996), it is not immediately clear which features of the tumor vasculature will provide the most appropriate targets for antitumor therapy, or whether these features will vary in different tumor types. This chapter focuses on recent results from studies in mice that begin to define the contributions of specific genes in diverse vascular systems, and the role of genetic diversity in the development of vascular patterning of normal and transformed tissues.

USE OF TRANSGENIC MICE TO STUDY THE IMPACT OF INDIVIDUAL GENES ON CANCER

Heterogeneity of genetic backgrounds, along with developmental age, diet, and a host of other variables, makes direct analysis of gene function a laborious and often imprecise task in humans and in cell lines. For this reason, genetically modified mice provide a unique tool to study the effects of specific genes on tumor growth

and on tumor-stromal interactions. Immune-competent mouse models offer the potential to study earlier stages of cancer progression than are possible in humans, and inbred mice offer additional advantages with respect to consistency of phenotype, unlimited access to tissue, and increasingly well-documented parallels to human development and physiology. Importantly, there are rapidly improving resources to identify genetic modifier loci in mice, which will prove critical in understanding genetic heterogeneity and its impact on disease. For specialized treatments of specific topics relevant to the production and analysis of tumor prone mice, several reviews are recommended (Bernstein and Breitman, 1989; Eastin et al, 1998; Macleod and Jacks, 1999; Tallquist et al, 1999; Cardiff et al, 2000).

Transgenic models of cancer provide opportunities to study (1) the effects of single or multiple mutations in all stages of disease progression in an immunocompetent host, (2) the effects of specific therapeutic agents or modifier genes on specific aspects of disease, and (3) the contribution of stromal cells (including endothelial, fibroblast, and immune cells) on tumor progression. While there are some limitations in the direct utility of mouse models for analysis of human genes, they have become an indispensable tool in efforts to improve the overall understanding of molecular mechanisms of disease.

While studies of human cell lines are of indispensable value to molecular studies of disease, the inheritance of multiple uncharacterized mutations in such cells and the limitation of ex vivo analysis make them relatively difficult to interpret in the context of cell behavior in vivo. Clearly, such studies are useful for establishing the effects of treatments on a limited range of cellular responses. Nonetheless, they lack the ability to evaluate the interactions of genes in an intact host and in a neutral genetic background. Eventually, there will be a need for researchers and clinicians to understand the genetic composition of both tumor and host/stromal cells and the interactions of genetic background in disease progression. One promise of the current explosion of information on molecular mechanisms underlying disease is that one may ultimately have access to better markers to define the status and therapeutic potential of specific disease states in individuals. In the case of tumor development, there is a growing appreciation of the diversity of mechanisms that can lead to tumor formation, and a realization that an equally diverse set of treatment options may ultimately be necessary to effectively regulate the process.

Gene targeting in transgenic mice, also referred to as reverse genetics, has progressed rapidly (Bernstein and Breitman, 1989). The ability to derive cells from any tissue that display altered expression of single gene has been a boon for the most advanced studies in every phase of disease. Of tumor-associated gene mutations alone, the opportunity to test specific hypotheses on cells overexpressing ras or epidermal growth factor (EGF) family members (Yuspa et al, 1994; Kim and Muller, 1999), or lacking only p53, *adenopolyposis coli* (APC), or a host of other genes (reviewed in Macleod and Jacks, 1999), has revolutionized our capacity to understand disease pathways. Studies of genetically altered mice have facilitated the first insights into mammalian tumor modifier genes (Parangi et al, 1995; Lifsted et al, 1998). In addition to the methodologies for specifically introducing and removing genes from the entire genome, the use of sequence-specific recombinases (cre and flp) have allowed the targeting of these effects in a manner that was not previously possible (Sauer, 1998).

The use of genetically defined, immunocompetent model mice will ultimately permit the types of rigorous studies needed to define issues related to genetic back-

ground and tissue heterogeneity on gene function. This chapter describes some of the resources available, the types of data that have been collected from transgenic mice for dissecting the complex interactions between endothelial cells within the tumor microenvironment, and some recent results on the diversity of angiogenic responses that can be observed within transgenic models of breast cancer in the context of transgenic mice.

THE USE OF TRANSGENIC MICE FOR THE ANALYSIS OF ANGIOGENESIS

Transgenic mice are animals whose DNA has been modified using molecular biology methods and who pass this modification to progeny. The current nomenclature distinguishes transgenic mice generated by random integration, TgN (insertional transgenics), from those generated by targeted integration, TgH (homologous recombination, "knockout," or "knockin"). An extensive discussion of the nomenclature and available lines of mice can be accessed through several Web-based search engines:

1. Genetic nomenclature for mice (http://www.informatics.jax.org/mgihome/nomen/)
2. TBASE (http://tbase.jax.org/)
3. Mouse genome informatics (http://www.informatics.jax.org/)
4. Transgenic database (http://www.biomednet.com/db/mkmd)
5. Internet resources (http://tbase.jax.org/docs/databases.html)
6. More internet resources (http://darwin.ceh.uvic.ca/bigbluc/transsites.htm)

A recent survey of these sites identified some 239 publications on mice engineered to overexpress known or putative oncogenes, and 259 publications on mice carrying mutant alleles of specific tumor suppressor loci. While the analysis of angiogenesis in transgenic tumor-prone mice has been relatively neglected, interest in this area is growing rapidly (Table 5.1, part A). This pioneering work has been led by D. Hanahan and colleagues, who have performed extensive analysis of tumor angiogenesis in transgenic models of skin and pancreatic cancer progression (Christofori and Hanahan, 1994; Hanahan and Folkman, 1996). Also evident, are the growing number of targeted deletions of genes thought to regulate vascular development. Some notable examples are included in Table 5.1, part B.

Because of the use of inbred lines, transgenic mice present a homogeneous genetic background in which to assess the involvement of specific genes. One can go on to investigate the interactions of genes by mating lines that contain single mutations and then analyze the phenotype of bigenic offspring. Over 100 different strains of transgenic mice have been generated carrying alterations in genes that affect vascular parameters, and well over 400 have been made involving genes that affect tumor predisposition. Thus, there is considerable opportunity, largely untapped, to study angiogenic parameters and therapeutic strategies in these animals.

While some of the phenotypes listed in Table 5.1 might not seem surprising, many were completely unexpected. The requirement for arylhydrocarbon

TABLE 5.1. Some transgenic models in which the vasculature* has been studied.

Transgene	Allele type	Phenotype	Tumor analysis†	Reference‡
(A) Overexpression phenotypes				
Angiogenin-1	TgN	AS	ND	(Suri et al, 1998)
Angiogenin-2	TgN	Disruption of emb. vasc.	ND	(Maisonpierre et al, 1997)
erbB2/neu	TgN	AS, TP	Yes	(Di Carlo et al, 1999)
FGF-2	TgN	AS	ND	(Fulgham et al, 1999)
fps/fep	TgN	AS, hemangiomas	Yes	(Green et al, 2000)
MMP-3 (activated)	TgN	AS, TP	Yes	(Sternlicht et al, 2000)
Neuropilin	TgN	AS	ND	(Kitsukawa et al, 1995)
v-*src*	TgN	AS, TP	Yes	(Theurillat et al, 1999)
Sv40T antigen	TgN	AS, TP	Yes	(Bergers et al, 1999) (Ganss and Hanahan, 1998)
Thrombospondin-1	TgN	AI		(Iruela-Arispe et al, 1999)
			Yes	(Ortega and Iruela-Arispe, submitted)
VEGF	TgN	AS, TP	Yes	(Larcher et al, 1998) (Zeng et al, 1998) (Detmar et al, 1998)
(B) Loss-of-function phenotypes				
Angiopoitin-1/2	TgH	Def. emb. vasc.	ND	(Suri et al, 1996)
ARNT	TgH	Def. emb. vasc.	ND	(Kozak et al, 1997)
		Def. VEGF expression		(Maltepe et al, 1997)
bmp4	TgH	EC overgrowth	ND	(Lawson et al, 1999)
crebbp	TgH	Def. vasc. remodeling	ND	(Oike et al, 1999)
dHAND	TgH	Def. emb. vasc.	ND	(Yamagishi et al, 2000)
EphB2/B3 Rc	TgH	Def. angiogenesis	ND	(Adams et al, 1999) (Wang et al, 1998a)
endoglin	TgH	Def. emb. vasc. remodeling	ND	(Arthur et al, 2000) (Li et al, 1999)
epo	TgH	Def. cardiac vasc.	ND	(Wu et al, 1999)
factor VII	TgH	Vasc. hemorrhage	ND	(Rosen et al, 1997)
FGF-1/FGF-2	TgH	No vascular defects	ND	(Miller et al, 2000)
Fibronectin	TgH	EC migration defects	ND	(George et al, 1997)
HIF-1α	TgH	Def. hypoxia response	Yes	(Carmeliet et al, 1998)
Integrin α_v	TgH	Brain vasc. def.	ND	(Bader et al, 1998)
Integrin α_4	TgH	Pericardial vasc. def.	ND	(Yang et al, 1995)
Integrin β_1	TgH	Teratomas w/ smaller vessels	Yes	(Block and Poncz, 1995)
Id1/Id3	TgH	Def. angiogenesis	Yes	(Lyden et al, 1999)
Lmo2	TgH	Def. large vessels/ remodeling	ND	(Yamada et al, 2000)
MEF-2C	TgH	Def. vasculature	ND	(Bi et al, 1999)
Mesp	TgH	Def. heart formation	ND	(Saga et al, 1999)
mek-1	TgH	Placental vasc. def.	ND	(Giroux et al, 1999)
myb	TgH	No vasc. def.	ND	(Krause et al, 1998)
Neuropilin-1	TgH	Def. yolk sac and large vessels	ND	(Kawasaki et al, 1999)
eNOS/iNOS/nNOS	TgH	Role for eNOS and nNOS in vasc. tone		(Fagan et al, 1999a)
		Hyperresponsive to hypoxia		(Fagan et al, 1999b)
p53	TgH	No vasc. phenotype	Yes	(Tyner et al, 1999) (Wang et al, 1998b)

TABLE 5.1. *Continued*

Transgene	Allele type	Phenotype	Tumor analysis*	Reference‡
PDGF-B and Rcβ	TgH	Def. pericytes/ hemorrhage	ND	(Lindahl et al, 1997)
prox1	TgH	Def. lymph angiogenesis	ND	(Wigle and Oliver, 1999)
sapin	TgH	Def. placental and heart EC	ND	(Sapin et al, 1997)
SCL/Tal1	TgH	Def. vasc. remodeling	ND	(Visvader et al, 1998)
smad 5	TgH	Def. vasc.	ND	(Yang, 1999)
TGF-β	TgH	Def. vasc.	ND	(Martin et al, 1995)
TGF-βRc type II	TgH	AS, TP	Yes	(Go et al, 1999)
TSP-1	TgH	Inc. vascularity	ND	(Lawler et al, 1998)
TSP-2	TgH	Inc. vascularity in dermis	ND	(Kyriakides et al, 1998)
Tissue factor (TF)	TgH	Def. emb. vasc.	ND	(Carmeliet et al, 1996)
TFPI	TgH	Emb. vasc. hemorrhage	ND	(Huang et al, 1997)
Tie-1	TgH	Vasc. maturation/ hemorrhage	ND	(Sato et al, 1995)
Tie-2/Tek-1	TgH	Def. vascular remodeling	ND	(Sato et al, 1995) (Dumont et al, 1994)
TNC	TgH	No vascular defect	Yes	(Talts et al, 1999)
VCAM	TgH	Def. emb. vasculature	ND	(Kwee et al, 1995; Terry et al, 1997)
VE-CAD	TgH	Def. vasc. remodeling	ND	(Carmeliet et al, 1999a)
VEGFA	TgH	Def. angioblast development		(Carmeliet et al, 1999b)
		Role in ras mediated tumors	Yes	(Grunstein et al, 1999)
VEGFRc1/flt-1	TgH	Def. emb. vasculature remodeling	ND	(Fong et al, 1995)
VEGFRc2/flk-1	TgH	Def. yolk-sac blood vessels	ND	(Shalaby et al, 1995)
VEGFRc3	TgH	Def. capillary remodeling	ND	(Dumont et al, 1998)
(C) Endothelial promoters characterized in mice				
VE-CAD	TgN	Ubiquitous EC expression		(Gory et al, 1999)
Tie-1	TgN	Heterogeneous EC expression		(Sato et al, 1995) (Korhonen et al, 1995)
Tie-2/Tek-1	TgN	Ubiquitous EC expression		(Schlaeger et al, 1997)
endothelin1	TgN	EC and some SMC expression		(Harats et al, 1995)
vWF	TgN	Heterogeneous EC		(Aird et al, 1995)
ICAM-2	TgN	EC, neutrophils, monocytes		(Cowan et al, 1996)
Flk-1	TgN	EC, angioblasts		(Kappel et al, 1999)

* Genetically manipulated inbred mouse strains with intact immune systems.

† Was the role in tumor development accessed?

‡ See additional references cited by the authors.

AS, angiogenic stimulation; AI, inhibition of angiogenesis; def., defect; EC, endothelial cell; emb., embryonic; ND, not determined; SMC, smooth muscle cell; TgN, transgenic overexpression; TgH, targeted mutation introduced by homologous recombination; TP, tumor promotion; TI, tumor inhibition; vasc., vasculature.

receptor nuclear translocator (ARNT) and its dimerization partner hypoxia-inducible factor-1α (HIF-1α) to mediate hypoxia-induced vascular endothelial growth factor (VEGF) induction (Maltepe et al, 1997; Carmeliet et al, 1998) is a prime example of a pathway defined through gene ablation, and has clear potential as a therapeutic target. Also surprising, yet informative, are the unexpected vascular phenotypes of transcription factors such as prox1, Id1, Id3, dHAND, and the lack of significant vascular phenotypes in fibroblast growth factor-1 (FGF-1), FGF-2, and p53 knockout mice. Of particular value, has been the growing appreciation for the diversity of genes that regulate vascular patterning. Loss of transcription factor prox1 ablates lymph-angiogenesis specifically (Wigle and Oliver, 1999), while the growth factor angiopoietin-1 (Suri et al, 1996) and its receptor Tie-2 (Dumont et al, 1994; Sato et al, 1995) are required for hierarchical vascular structures and vascular remodeling, but not for earlier establishment of the embryonic vasculature. One clear take-home message from these studies is the potential resource that transgenic animals provide in isolating cell types, such as endothelial and smooth muscle cells, that lack specific gene products. These cells can be used to define biochemical pathways such as the requirement of urokiuase-type plasminosen activator (uPA) in activation of L-transforming growth factor-β_1 (L-TGF-β_1) (Herbert and Carmeliet, 1997). Although the effects of many of the induced mutations have yet to be analyzed in the context of tumor promotion, there is a clear expectation that such analysis could provide insight into pathways that hold promise for therapeutic manipulation. In those cases in which null mutations lead to embryonic phenotypes, such analysis will have to await the development of tissue-specific or inducible deletions of the genes in question, which could facilitate investigations of adult phenotypes. A large literature has developed on such systems using sequence-specific recombinases such as cre (Sauer, 1998) and flp (Vooijs et al, 1998). By linking expression of cre-recombinase to endothelial-specific promoters (Table 5.1, part C), several genes have already been ablated from specific vascular cells. These include VEGF (Grunstein et al, 1999) and vascular cell adhesion molecule-1 (VCAM-1) (Terry et al, 1997). Drug-inducible forms of cre and flp seem to be particularly powerful tools for analyzing the role of essential genes in many physiologic states that are not currently accessible by standard targeting strategies (Brocard et al, 1998; Sawicki et al, 1998).

GENETIC MODIFIERS OF ANGIOGENESIS AND VESSEL MORPHOLOGY

There is very little information on classic modifier loci that directly influence angiogenesis; however, major inroads into this potentially important area are beginning to be made. Strain-specific susceptibility to cancer formation in inbred mice has been used to identify loci that affect a variety of tumor phenotypes, such as susceptibility to colon cancer (Cormier et al, 1997; Dove et al, 1998) and retinoblastoma (Griep et al, 1998). However, the identity of modifiers in tumor angiogenesis in these models is currently unknown.

That genetic variants play a role in vascular phenotypes is no longer in doubt. Recently, D'Amato and colleagues have demonstrated large genetic contributions to the response of mice to the angiogenic stimulators FGF-2 and VEGF (Rohan et al, 2000). They demonstrated a 10-fold difference in the response of 129/SvImJ

mice and C57BL/6J to FGF-2 in corneal pocket assays. Furthermore, they also found strong evidence of genetic modifiers of the antiangiogenic factors thalidomide and TNP-470. While the D'Amato study is the clearest evidence that genetic modifiers are involved in the magnitude of the angiogenic response, other studies on the genetic susceptibility of mice to tumor progression may also uncover modifiers of this class. Studies that have mapped tumor susceptibility in lung cancer have shown that different modifiers affect tumor initiation and progression. Coussens and colleagues (1996) showed extensive strain differences to tumor progression of squamous carcinomas that develop in the K14-HPV16 transgenic mice. Finally, there is evidence of alleles of a locus on chromosome 4 that predispose to large tumor size in a lung tumor model (Manenti et al, 1997). If modifiers were identified that affect the vascularization of tumors, it is likely that they would be of a class that influences tumor progression as opposed to initiation. Thus, one could potentially devise screens to isolate such variants. With rapid improvement in the tools available for genetic analysis in mice (Macleod and Jacks, 1999), it is very likely that steady gains will be made toward identifying these and other genetic modifiers of angiogenesis.

BLOOD VESSEL RECRUITMENT AND THE ROLE OF MOUSE MODELS IN GENETIC ANALYSIS OF TUMOR ANGIOGENESIS

The mechanism of blood vessel recruitment in tissues has been extensively investigated in mouse models. From analysis of knockout mice, it is clear that VEGF and angiopoietin-1 (Ang-1) act as principal positive comodulators of angiogenesis (see Chapter 2). Conversely, Ang-2 and thrombospondin-1 (TSP-1), are emerging as potent negative regulators (Iruela-Arispe and Dvorak, 1997; Maisonpierre et al, 1997). Targeted gene inactivation studies in mice have shown that VEGF is necessary for very early stages of vascular development, whereas Ang-1 is required for vascular remodeling at later stages (Suri et al, 1996; Carmeliet and Collen, 1999). In addition, mouse models have provided extensive support for the angiogenic-switch hypothesis, which suggests the solid tumors progress from avascular, to vascular stages, in discrete genetic steps (Folkman and Hanahan, 1991; Hanahan and Folkman, 1996). New evidence suggests a potential role for other additional mechanisms of vascular recruitment in tumors such as "co-opting" of existing blood vessels (Holash et al, 1999a,b). Such alternative mechanisms have yet to be demonstrated in model systems, and will undoubtedly benefit from more extensive molecular characterization.

Angiogenic regulators include a variety of primary oncogenes and tumor suppressor genes as well as genetic modifier genes that affect general angiogenic responsiveness within an individual. Growth factors such as TGF-α, EGF, platelet-derived growth factor-BB (PDGF-BB), TGF-β, and several FGF family members (HSTF1, int2) are primary oncogenes that also enhance the recruitment of vascular endothelial cells (Folkman and Klagsbrun, 1987). TSP-1 is currently the most important of the classic tumor suppressor genes known to have direct antiangiogenic activity in tumors (Good et al, 1990; Streit et al, 1999). In addition, it is becoming increasingly clear that a large number of tumor genes regulate the production of angiogenic factors (Rak et al, 1995; Ravi et al, 2000), and thus

regulate angiogenesis directly or indirectly. Many oncogenic signals must now be considered as part of the machinery that controls capillary recruitment and maintenance.

As a primary example of this phenomenon, there is growing evidence that VEGF expression is variable in tumors (Brown et al, 1995; Kvanta et al, 1996) and is dependent on both hypoxia (Shweiki et al, 1992) and the expression of particular oncogenes. VEGF expression is highly upregulated in cells transformed by mutated *ras* (Rak et al, 1995; White et al, 1997), v-*src*, and mutated *p53* (Mukhopadhyay et al, 1995), but is not particularly abundant in cells transformed with ornithine decarboxylase (Auvinen et al, 1997). Conversely, by using mouse cells engineered to lack VEGF expression, it has been shown that oncogenic *ras* requires VEGF for transformation. Indeed, VEGF deficient embryonic stem cells expressing *ras* formed small poorly vascularized tumors, providing evidence that VEGF induction is crucial for effective tumor growth in vivo (Shi and Ferrara, 1999). It has also been demonstrated that loss of VEGF expression results in decreased vascular density and permeability, and leads to tumor cell apoptosis (Grunstein et al, 1999), which corroborates the role of VEGF in tumor angiogenesis. Mutations in the tumor suppressor gene *p53* are commonly found in a wide variety of human tumors. Interestingly, there is some evidence to suggest that wild-type *p53* plays a role in angiogenesis by suppressing basal VEGF production (Mukhopadhyay et al, 1995). It has also been shown that transfection of mutant *p53* is able to induce expression of VEGF messenger RNA (mRNA) (Kieser et al, 1994), suggesting that *p53* gene may have a role, directly or indirectly, in regulating VEGF expression.

ANALYSIS OF ANGIOGENIC REGULATORS IN TRANSGENIC TUMOR MODELS IN THE MAMMARY GLAND

While an extensive literature has developed on the ability of primary oncogenes to stimulate breast cancer progression in transgenic mice (Cardiff et al, 2000), few studies have specifically addressed angiogenic parameters within these tumors, or the response of the tumor vasculature to diverse stimuli. Thus, mammary tumors in transgenic mice represent a relatively untapped resource for understanding the vascular biology of mammary tumors.

Mice carrying *erbB2* transgenes e.g., mouse mammary tumor virus (MMTV)-driven *neu* have been widely used because of the short latency and high penetrance of mammary tumor formation. Treatment of the MMTV-*neu* mice with interleukin-12 (IL-12) appeared to inhibit early stages of tumor formation while having less effect on late-stage, more developed, tumors (Boggio et al, 2000). The authors tested the effects of delivering similar doses of IL-12 spaced over different time courses and saw similar effects in mice from two different genetic backgrounds carrying different activating mutations of the *erbB2/neu* oncogene.

In a second example, the effects of metalloproteinase inhibitors has also been analyzed due to the expectation that neoangiogenesis relies heavily on matrix remodeling factors. Coexpression of TIMP-1 (tissue inhibitor of metalloproteinases) has now been shown to inhibit tumor progression in two independent transgenic models. The Khokha lab has shown that regulation of TIMP-1 has reci-

TABLE 5.2. Analysis of angiogenic effectors in tumor-prone transgenic mice.

Transgene	Agent	Dosage	Effect		Reference
			Tumor growth	Angiogenesis	
MMTV-TGF-α	TGF-β	TgN	Inc.TFS, dec.TM	ND	(Pierce et al, 1995)
Insulin-Sv40T	TNP-40*/IFN	var.	Dec.TV	Dec.	(Bergers et al, 1999; Parangi et al, 1996)
MMTV-neuT	thbs1	TgN	Inc.TFS, dec.TM	Dec.	(Iruela-Arispe et al, 1999)
MMTV-TGF-β	L-744,832†	Var.	Inc.TFS, dec.TM	ND	(Norgaard et al, 1999)
H2-Sv40T	TIMP1	TgN	Dec.TV	Dec.	(Martin et al, 1999)
MMTV-neuT	IL12	var.	Inc.TFS, dec.TM	Dec.	(Boggio et al, 2000)
MMTV-neuN	IL12	var.	Inc.TFS, dec.TM	Dec.	(Boggio et al, 2000)
C3(1)-Sv40T	IL12/IL2	var.	Dec.TV	Dec.	(Green et al, 2000)

* Fumagillin analog.

† Inhibitor of farnesyl-protein transferase.

var., various; inc., increase; dec., decrease; TgN, inhibitor was delivered as a transgene; TFS, tumor free survival; TM, tumor multiplicity; TV, tumor volume.

procal effects on hepatic tumor progression (Martin et al, 1999) and that this effect is, at least partially, related to inhibition of angiogenic parameters in the tissues. Stromelysin-1 (MMP-3), a target for TIMP-1, can induce mammary carcinogenesis when overexpressed in mammary epithelial cells (Sympson et al, 1995; Witty et al, 1995; Thomasset et al, 1998). Coexpression of TIMP-1 and MMP-3 in double transgenic animals blocked tumor progression (Sternlicht et al, 2000).

Though the transformed phenotype is recognized to be complex in all transgenic tumor prone mice, it is clear that use of such animals will have enormous value for assessing the contribution of angiogenesis, and other phenotypes, in tumor progression. Table 5.2 lists a few additional examples of the types of transgenic systems that have been used to study angiogenic recruitment in genetically defined tumor systems.

EVIDENCE THAT TUMOR VASCULATURE IS MODULATED BY THE INITIATING ONCOGENIC EVENTS IN THE MAMMARY GLAND

Although little is known about the regulators of tumor morphology, a considerable amount of research has been focused on establishing a functional link between genetic alteration(s) and acquisition of angiogenic properties. To examine whether tumor genetics could be predictive of vascular patterning, we examined the tumor vasculature of a variety of breast cancer prone transgenic mice.

The studies were performed on a series of transgenic mice that mimic aspects of human breast cancer progression by overexpression of oncogenes (*erbB2/neu*, *wnt10b*), by underexpression of specific tumors suppressor genes (*Brca1* or *p53*), or by overexpression of tumor suppressor genes (*TGF-β*) implicated in this disease. The lines that we will discuss (Table 5.3) include animals that (1) overexpress an activated form of *erbB2/neu* under control of the MMTV promoter (NK) (Muller et al, 1988); (2) overexpress the oncogenic growth factor *wnt10b* under

Table 5.3. Transgenic models used for the analysis of vascular morphology in mammary tumor development.

Transgene	Line	Phenotype	Reference
MMTV-neuT	NK	Rapid transformation	(Muller et al, 1988)
p53 null	BB	Genomic instability	(Donehower et al, 1992)
MMTV-wnt10b	WA	Rapid transformation	(Lane and Leder, 1997)
MMTV-Brca1 antisense	BAS	Genomic instability	(Lane and Leder, in preparation)
MMTV-TGF-β	TB	Tumor suppression	(Pierce et al, 1995)

control of the MMTV promoter (WA) (Lane and Leder, 1997); (3) carry a null mutation in the p53 locus (BB) (Donehower et al, 1992); (4) have reduced levels of *Brca-1* in mammary tissues resulting from expression of *Brca1* antisense RNAs (BAS) (Lane and Leder, in preparation); or (5) overexpress the *TGF-β* tumor suppressor gene in the context of *wnt10b* tumors (Pierce et al, 1995).

The transgenic strains have been created in the FVB/n strain or back-crossed for >12 generations into FVB/n. FVB/n is an inbred strain of mice that provide a powerful tool for genetic analysis as they have good parenting behavior, large litter sizes of 10 to 12, and display good sensitivity to a variety of oncogenic stimuli (Taketo et al, 1991). A recent study comparing the response of several strains to angiogenic stimulation by FGF-2 and VEGF placed FVB/n as intermediate in response to both factors (Rohan et al, 2000).

While dissecting tumors from these mice, it became evident that some tumors appeared more reddish and/or bled more profusely than others. These features were strongly correlated with the initiating genetic mutation, but appeared independent of size, time of onset, or whether the tumors were primary, metastastic, or had been transplanted as single cell suspensions into syngeneic hosts.

Histologic examination of tumors showed distinct, oncogene-specific, patterns of tumor cell organization, as has been described previously (Cardiff et al, 1991). Vascular differences were also evident from these preparations (Figure 5.1), particularly as to whether the tumors showed thin capillaries irradiating into a compact mass of cells (e.g., NK or WA alone), or distended vessels and lacunae (e.g., WA × TGF-β).

As seen in Figure 5.1, three-dimensional (3D) reconstruction of tumor vasculature demonstrates significant differences in density and fine structure in different transgenic tumors. Precautions were taken to allow fair comparison between lines: (1) Confocal micrographic analysis was carried out using the same scanning parameters (magnification, thickness, laser intensity, reconstruction analysis). (2) Since tumors varied in the time of incidence, age of animals could not be matched; we chose to normalize our analysis to tumors of 0.8 to 1.2 cm^3. (3) Several animals (three to nine) were evaluated from each tumor model to ensure reproducibility of the pattern. (4) The area scanned was identical. (5) The area used to perform the scan was exactly 100 μm below the capsule of the tumor (Figure 5.1 inset).

From the samples imaged in Figure 5.1, vascular density (VD) and vascular volume (VV) were calculated using ImagePro software algorithms. VV is a measure of vessel capacity, which can be very informative. For instance, although WA × TGF-β tumors had lower vascular count (VD), the overall capacity of this vascular bed exceeds that of tumors expressing WA alone by 2.8-fold. Therefore, we

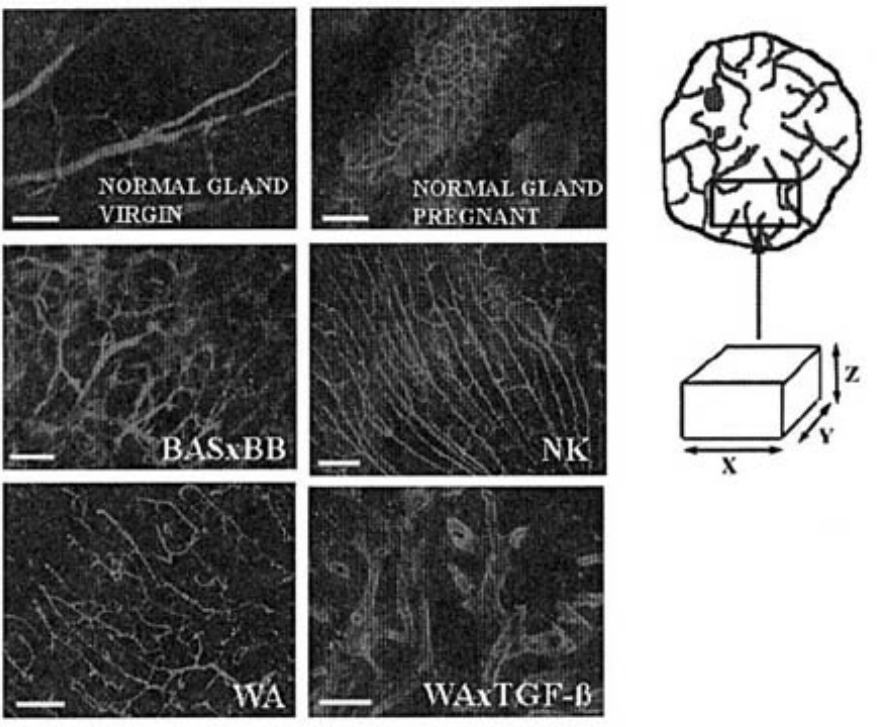

FIGURE 5.1. Genetic differences in initiating oncogenes leads to reproducible differences in vascular architecture. Syngeneic transgenic mice, carrying different initiating oncogenic mutations, were followed until mammary tumors developed to 1.0 cm^3. The mice were perfused with fluorescein isothiocyanate (FITC)-lectin conjugates and then perfusion fixed with buffered paraformaldehyde (4%) prior to analysis of vascular pattern by confocal microscopy. Panels include vascular profiles from normal and pregnant mammary glands, as well as from BAS (*BRCA-1* deficient), BB (*p53* deficient), NK (activated *erbB2/neu*), WA (*wnt10b* activated), and transforming growth factor-β (TGF-β) overexpression. The images represent confocal reconstructions of 3D images collected as described in the cartoon, and in the text.

found that measures of capillary density alone provide incomplete information as to vascular volume (capacity). When similar morphometric measurements were performed on a variety of tumors arising in distinct transgenic lines, we observed consistent differences in the degree and pattern of vasculature, that was predictive of the initiating tumorgenic mutation (Table 5.4).

These results show that simple counts of capillary profiles underestimate true vessel counts and that differences in vascular pattern were only clearly discernible by 3D reconstruction. Although traditional sectioning and staining for CD-31 (or other comparable endothelial markers) gave similar overall results with respect to vascular density (not shown), three limitations of this type of analysis became evident from our experiments: (1) many tumor capillaries do not uniformly express CD-31 or CD-34; (2) quantification of vascular density and volume from standard sections is more time-consuming, less reproducible, and prone to fixation and embedding artifacts; and (3) information concerning branching, network structure, and hierarchy is laborious or impossible to obtain from sections.

TABLE 5.4. Summary of tumor associated vascular patterns emerged from the analysis.

Tumor genotype	Observed pattern of tumor vasculature
BAS* × BB	Hierarchy in vessel diameter was preserved; vascular fragility was noted, areas of vessel rupture were evident, frequent branching and capillary networks were observed
NK	Long, thin vessels were observed with frequent branching; the hierarchy of vessel sizes was partially affected
WA	Wandering vessels were observed with frequent branching; the hierarchy of vessel sizes was partially affected
WA × TGF-β	Hierarchy largely lost, thick vascular channels less frequent than in WA alone.

* Indicated strains are described in Table 5.3 and in the text.

In summary, depending on the nature of the genetic transformation, we observed consistent changes in the vascular profile. These results support the idea of complex interaction between tumor cells and the tumor vasculature. Unlike previous analysis, 3D reconstruction demonstrated a clear link between vessel phenotype and the type of oncogenic transformation. Some of these changes related to changes in extracellular matrix proteins, particularly those associated with basement membrane structure (data not shown).

MODELS AND REPORTER SYSTEMS FOR ASSESSING ANGIOGENIC ACTIVITY IN MICE

Difficulties in identification of capillaries and small vessels provide a consistent limitation to quantitative analysis of angiogenesis in tissues. In particular, identification of vascular cells in tissue biopsies has been limited by availability of endothelial markers that are heterogeneously expressed in capillaries. As we have shown, such analysis is extremely limited in appreciating higher order vascular structures. In addition to 3D reconstruction of tumor explants, methodologic advances may soon provide access to such analysis noninvasively in the whole animal.

One approach is through the development of mice carrying "pre-labeled" endothelial cells. A particularly elegant example of this is use of autofluorescent proteins as reporter genes linked to endothelial-specific promoters. Brian Seed's group has shown the power of this approach by linking promoter elements from the VEGF gene to green fluorescent protein (GFP). Transgenic mice expressing this construct have been shown to produce fluorescent cells in response to anoxia and other proangiogenic cues (Fukumura et al, 1998). Such strategies could be linked to more sensitive and potentially less invasive visualization methodology.

Very promising avenues of research lie in the development of reporters that can be imaged in live animals. One avenue being developed by Michael Phelps and colleagues is the use of positron emission tomography (PET) instrumentation that can visualize small tumor masses in cells expressing transgenes (Gambhir et al, 1999; MacLaren et al, 1999). Though in its infancy, mathematical algorithms are being devised that may permit the analysis of bioluminescence directly in tissues. Luciferase reporter genes can be visualized in whole mice (Benaron et al, 1997).

This methodology has also been used to monitor adenoviral vector mediated gene expression in the mouse brain (Contag et al, 1997), and tumor cell clearance from therapeutically manipulated mice (Sweeney et al, 1999). Continued work in this area could refine these approaches from the current rather macroscopic limits. In the case of the new generation of micro-PET instruments, improvements in imaging reporters could bring resolution into the microliter (μm^3) range (Gambhir et al, 2000).

DISCUSSION

Though great strides have been made in identifying key regulators of angiogenesis, there is much more work to be done. The ability to effectively target mutations in mice is unsurpassed in any other vertebrate system, and affords the only method for analyzing the role of loss of function alleles in embryonic vascular development. This assay is becoming an essential tool for the analysis of gene function, and is particularly powerful in defining genetic pathways that regulate complex developmental events within the whole animal. Studies of transgenic animals are also facilitating advances in identification of efficacious therapeutic targets and in understanding the role of genetic modifiers. It appears fair to say that the impact of specific genes on angiogenesis, as opposed to other systems, depends critically on the ability to study the effects of loss-of-function mutations in mammalian development. The critical roles of the Tie receptor tyrosine kinases (Sato et al, 1995) and the more recent loss-of-function phenotypes of broadly expressed transcription factors like SMAD5 (Yang, 1999) and myocyte enhancer factor 2C (MEF) (Bi et al, 1999) would have been difficult to show without modern approaches to manipulating the genome of mice.

Given our data on the specific vascular phenotypes within tumors driven by diverse transforming genes, it is likely that much more work is needed to understand the role of vascular hierarchical structures in disease progression and therapy. Future efforts should focus more attention on the vascular phenotypes of transgenic models in which genetic background can be controlled. Because of their size and ease of manipulation, mouse models will play a major role in the development of new methodologies for less invasive high-resolution imaging of vascular development. Ultimately, all these approaches hold promise for moving the field toward a more comprehensive understanding of the molecular and cellular interactions that regulate the complex tissue interactions in animals and humans, and the critical role that angiogenesis plays in this process.

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