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Transgenic Mouse Models of Tumour Angiogenesis: the Angiogenic Switch, its Molecular Controls, and Prospects for Preclinical Therapeutic Models

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INTRODUCTION

THE ABILITY to genetically engineer the mouse has allowed the development of an increasing number of murine models of carcinogenesis [1–4]. The power of these models lies in their consistency of tumorigenesis, wherein malignancy develops in a specific tissue(s), at a predictable age, and in some instances, from a particular type of premalignant lesion. Historically, two approaches have been used to generate families of cancer prone mice that heritably recapitulate oncogenes from normality to cancer: direct injection of pronuclei of one-cell embryos with DNA encoding dominant oncogenes (producing 'transgenic mice'), or targeted disruption of tumour suppressor genes in embryonic stem cells which are subsequently injected into blastocysts (resulting in 'gene knockout mice'). These technologies are being further refined, with the emergence of techniques for tissue specific gene knockouts and postnatal induction, or repression, of transgene expression.

The reproducible induction of tumorigenesis in either transgenic or knockout mice has facilitated evaluation of the process of tumour development from normal cells in their natural tissue environment, revealing in many cases a multistep progression through discrete histological and temporal stages. Thus, in a number of cases, a series of premalignant stages has been identified, ranging from histological normality to hyperplasia, to varying degrees of dysplasia and carcinoma *in situ,* which appear prior to the emergence of full blown cancers. These multistage models possess several features which have enhanced investigation of carcinogenesis, including: (i) the ability to biopsy premalignant lesions routinely, even very early ones, for biochemical, physiological and molecular genetic analyses, and for derivation of representative cultured cell lines; (ii) assessment of genetic complementation of carcinogenesis by generating double transgenic or composite transgenic/knockout mice with altered expression of particular candidate genes; (iii) identification of genetic loci controlling stage-specific transitions by generating polymorphic genetic hybrids and constructing genetic and physical chromosome maps; and (iv) assessment of the patterns and role of particular biological processes, such as altered differentiation or programmed cell death, at each stage of carcinogenesis.

Among the biological parameters that are proving accessible for studying in transgenic or knockout murine models of cancer is the control of tumour angiogenesis and the assessment of its role in neoplastic progression and malignant conversion. This review summarises the early studies on tumour angiogenesis in selected transgenic murine models, and describes current studies which have set the stage for future research, both into mechanism and its application to therapeutics and prevention.

THE ANGIOGENIC SWITCH OCCURS DURING PREMALIGNANT STAGES OF TUMORIGENESIS

To date, vascularisation has been evaluated in three diverse murine models of tumorigenesis. These are: (1) pancreatic islet β cell tumours induced by targeted expression of *SV40 Tag* oncogene under control of the insulin gene regulatory region (in RIP-Tag transgenic mice) [5]; (2) dermal fibrosarcomas elicited by the bovine papillomavirus genome (in BPV1.69 transgenic mice) [6]; and (3) epidermal squamous cell carcinomas that arise from basal keratinocytes expressing the human papillomavirus type 16 oncogenes controlled by the keratin 14 gene regulatory region (K14-HPV16 transgenic mice) [7, 8]. In each case, a multistage pathway of tumour development is evident, histologically and temporally.

In the prototype model of islet cell carcinogenesis, the multifocal nature of the islets (400/pancreas) and the universal tumour penetrance by 12–14 weeks has facilitated extensive anatomical, biochemical and molecular analyses [5, 9–12]. The discrete stages of tumorigenesis in these RIP-Tag transgenic mice include an initial phase of oncogene expression without apparent consequence, beginning at embryonic day 8.5 extending to approximately 3 weeks of age. Then, hyperplastic islets sporadically begin to emerge; and eventually, approximately 50% of the islets become hyperproliferative. These focal nodules, historically referred to as hyperplastic islets, have the characteristics of a carcinoma *in situ,* in terms of cellular morphology and mitotic

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incidence. Unexpectedly, a subset (approximately 20%) of the hyperplastic islets subsequently become angiogenic, and, of these, approximately 10% progress to solid tumours [13]. The induction of angiogenesis has been documented by histopathology, and substantiated using an *in vitro* bioassay for angiogenesis involving co-culture with bovine capillary endothelial cells in collagen gels. Two important concepts emerged from this early characterisation of tumorigenesis in RIP-Tag transgenic mice: (i) discrete stages of premalignant progression were evident, a hyperplastic phase followed by a stochastic angiogenic stage, and (ii) neovascularisation developed well before the emergence of an invasive malignancy (Figure 1). These data suggested a hypothesis that the induction of neovascularisation during multistage carcinogenesis is co-ordinated by a discrete event, an 'angiogenic switch'.

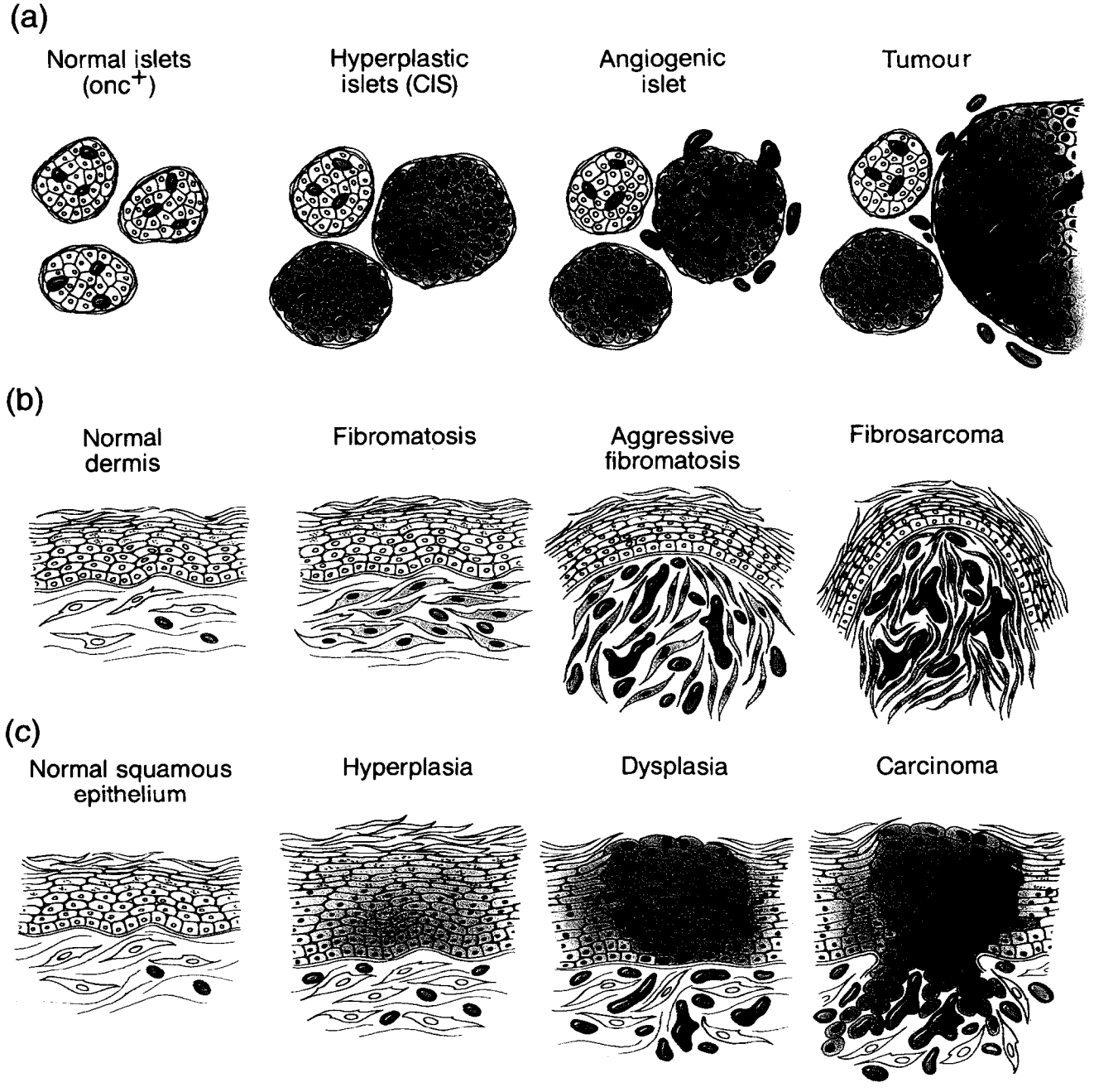

Figure 1. The angiogenic switch occurs prior to tumour formation in three transgenic mouse models of tumorigenesis. (a) Expression of the *Tag* oncogene in the pancreatic islets elicits four sequential stages in tumour development: 'normal', oncogene expressing islets; hyperplastic islets, populated by proliferating cells with the histological hallmarks of carcinoma *in situ;* angiogenic islets, wherein new blood vessel growth has been activated; and solid tumours, which are islet cell carcinomas. (b) In transgenic mice carrying the BPV-1 oncogenes, the normal dermis is initially converted into a state of mild fibromatosis, revealed as focal accumulation of dermal fibroblasts. Angiogenesis is first evident in the next stage, aggressive fibromatosis, which is also marked by dense arrays of proliferating fibroblastic cells; both hyperproliferation and angiogenesis persist in the subsequent stage, protuberant fibrosarcoma. (c) Targeted expression of the HPV16 oncogenes to basal cells of the epidermis induces multistage development of squamous cell carcinoma, beginning as hyperplasia of keratinocytes, with a mild increase in vessel density, which progresses to dysplasia, marked by morphologically aberrant keratinocytes with a high proliferation index, and by abundant neovascularisation; finally two classes of squamous carcinoma arise, both show extensive angiogenesis.

In the second transgenic model of multistage tumorigenesis, formation of dermal fibrosarcomas in BPV1.69 transgenic mice occurred in three distinct stages: normal, mild fibromatosis and aggressive fibromatosis. These stages are histologically distinct [14], and characterised, for example, by differential expression of the *c-jun* and *jun-B* proto-oncogenes and their associated AP1 transcription factor activities [15]. Characterisation of the density of the vasculature by immunostaining for von Willebrand's (vWF) factor revealed a dramatic increase in capillaries in the aggressive fibromatosis stage [16], again emphasising the activation of an angiogenic switch in a premalignant stage prior to the emergence of the fibrosarcomas (Figure 1). Assays of cell lines cultured from each stage of the progression to fibrosarcoma provided further evidence for the existence of a discrete angiogenic switch: conditioned medium from cells derived from normal dermis and mild fibromatosis did not contain endothelial cell mitogenic activity, whereas those from the aggressive fibromatosis and fibrosarcomas did, consistent with the histological evidence for vascular quiescence or angiogenesis, respectively, in their cognate lesions [16].

Evidence for activation of an angiogenic switch in premalignant lesions has also been obtained from a third model of multistage tumorigenesis, epidermal carcinogenesis in K14-HPV16 transgenic mice. Both well and moderately differentiated squamous cell carcinomas arise from pathways beginning as hyperplasia and progressing through varying degrees of dysplasia [7, 8, 17, 18]. A perceptible increase in dermal capillary density is first apparent in the hyperplastic stage of one month old transgenic mice. There is a striking increase in both the number and distribution of dermal capillaries in the early and advanced dysplastic lesions; numerous vessels become closely apposed to the basement membrane separating dysplastic keratinocytes from the underlying stroma (K. Smith-McCune, University of California, San Francisco, U.S.A.). The pattern is indicative of an angiogenic switch from vascular quiescence to an initial condition of modest neovascularisation seen in the early low grade lesions, followed by a second, striking upregulation of angiogenesis in high-grade neoplasias as well as in the invasive cancers (Figure 1).

Thus, three distinctive transgenic mouse models of tumorigenesis indicate an angiogenic switch to new blood vessel growth beginning well before the emergence of solid tumours (the pathways are compared schematically in Figure 1). A similar pattern of activation of angiogenesis is evident in a subset of premalignant lesions associated with, and ascribed as stages in the genesis of, several human cancers, including: dysplastic melanocytic lesions (naevi) thought to precede malignant melanoma (reviewed in [19]), mammary ductal carcinoma *in situ* seen in association with invasive breast carcinomas, and moderate to high-grade cervical dysplasias implicated as progenitors of uterine cervical carcinoma (reviewed in [20]). Collectively, the investigations of both murine models and human diseases suggest that premalignant activation of angiogenesis is a general parameter of tumour development and predicts early activation of an angiogenic switch in additional examples of multistage carcinogenesis in humans and other mouse models. As such, this hypothesis indicates that subsequent investigations should focus on both the mechanism of the switch, and on novel potential antineoplastic therapies and/or regimens of chemoprevention. The following sections address these issues from the perspective of prototype multistage transgenic mouse models activating an angiogenic switch during tumorigenesis.

ANGIOGENESIS INDUCERS: CONTRASTING TUMOUR-SPECIFIC PATTERNS OF EXPRESSION

Historically, the angiogenic switch, whereby the normally quiescent vasculature grows new capillaries, has been envisioned to be activated by the synthesis or release of angiogenic growth factors. This proposition was based on the capability of tumours and tumour cells to elicit the ingrowth of capillaries when placed into avascular rabbit cornea or the characteristically vascularised chick chorio-allantoic membrane. The discoveries that angiogenesis can be activated by acidic and basic fibroblast growth factors (a/bFGF) and by vascular endothelial growth/permeability factor (VEGF/VPF), strengthened the notion of paracrine induction of angiogenesis by peptide growth factors. Subsequently, a/bFGF and VEGF have been found to be upregulated in a variety of malignancies and derivative malignant cell lines, presenting further circumstantial evidence for an important role of inducer molecules in the activation of angiogenesis (discussed elsewhere in this Issue, and in recent reviews [20–25]).

Access to premalignant stages of tumorigenesis inherent in transgenic murine models has uncovered two distinctive patterns of expression of angiogenesis regulatory molecules: one is consistent with the model suggested by angiogenesis bioassays, that upregulation of inducer gene expression could serve to activate the switch; the second shows constitutive expression of angiogenesis inducer genes during both vascular quiescence and angiogenesis, implicating alternative mechanisms for activating the switch.

The first pattern of upregulation of angiogenesis-inducer genes, is evident during epidermal squamous carcinogenesis in the K14-HPV16 transgenic mice (Figure 2) [26], (K. Smith-McCune, University of California, San Francisco, U.S.A.). In normal murine epidermis, and in 'normal' epidermis expressing the HPV16 oncogenes, neither aFGF nor VEGF are transcribed at detectable levels. Expression of each, as shown by RNA *in situ* hybridisation, is barely detectable in early stage hyperplastic transgenic epidermis. There is a notable increase in aFGF and VEGF mRNA levels in high-grade dysplastic premalignant precursor lesions. There is marked concordance of aFGF and VEGF upregulation with induction of angiogenesis revealed by histopathology and vWF immunostaining in dysplasias, suggesting that the increasing levels of these two inducer molecules activate the angiogenic switch to progressively higher intensity settings. bFGF shows the contrasting pattern: expression is detectable in normal epidermis, persisting at similar levels in hyperplasia, dysplasia and moderate-poorly differentiated cancers. Modest upregulation is seen in well-differentiated carcinomas. This constitutive expression of the bFGF gene by keratinocytes may produce levels of inducer protein insufficient to activate the capillary endothelium to the angiogenic phenotype; subsequent increases in inducer levels afforded by VEGF and aFGF upregulation might then produce levels that 'turn on' the switch. Alternatively, additional factors, such as sequestration of bFGF or counterbalancing angiogenesis inhibitors,

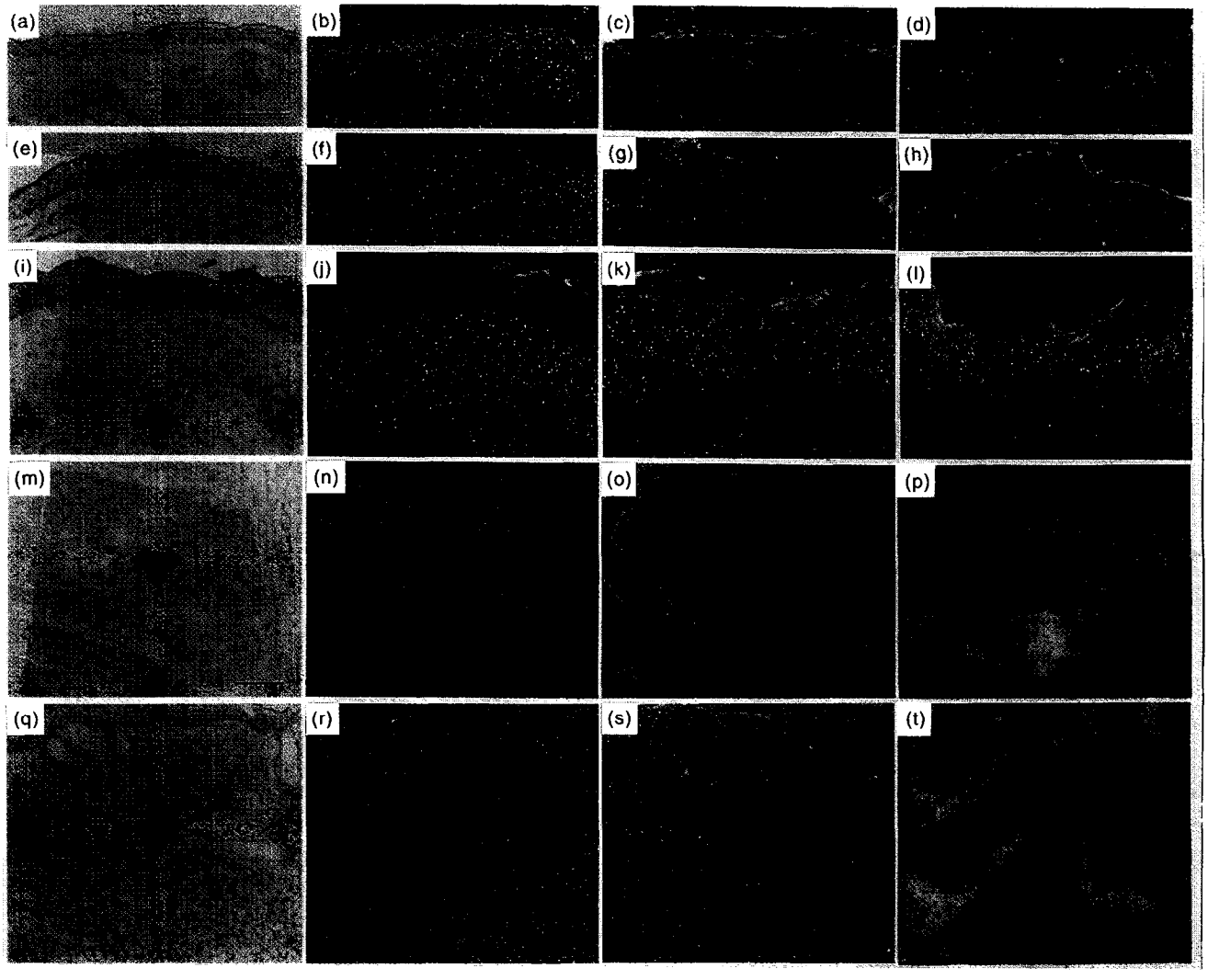

Figure 2. Expression of bFGF, aFGF and VEGF during epidermal squamous carcinogenesis in K14-HPV16 transgenic mice as determined by 35_S mRNA *in situ* hybridisation (ISH). These angiogenesis inducers display two distinct patterns of expression during carcinogenesis. The left column shows brightfield sections from normal non-transgenic epidermis (a), hyperplasia (e), dysplasia (i), well-differentiated (m), and moderate-poorly (q) differentiated epidermal squamous cancers. The second column shows that bFGF is constitutively expressed in both normal, non-transgenic epidermis (b), and in the premalignant hyperplastic (f) and dysplastic (j) stages. bFGF expression appears to increase somewhat in well differentiated epidermal cancers (n), but remains at levels similar to dysplasia in the moderate-poorly differentiated malignancies (r). Both aFGF (third column) and VEGF (right column) are induced during carcinogenesis. Expression of both growth factors are undetectable in normal, non-transgenic epidermis (c, d). There is perceptible induction of aFGF and VEGF in hyperplastic epidermis (g, h); with a further increase in the dysplastic stages (k, j). Following malignant conversion, aFGF mRNA remains at a modest level, comparable to dysplasia (k) in both well (o) and moderate-poorly differentiated epidermal cancers (s), whereas there is a striking further increase in VEGF mRNA in well differentiated (p) and moderate-poorly differentiated (t) cancers. Bars: A–L = 20 μm; M–T = 50 μm

may be involved in the non-angiogenic stages, as discussed in the sections below.

The contrasting situation of constitutive expression of angiogenesis inducers has been revealed by the analysis of FGF and VEGF expression patterns during multistage tumorigenesis in the islet cell carcinoma model. Surprisingly, we observed that both VEGF [27] and aFGF (G. Christofori, Research Institute of Molecular Pathology, Vienna, Austria) are expressed at high levels in normal pancreatic islets of control non-transgenic mice, at the RNA (not shown) and protein levels (Figure 3). The VEGF receptor genes *flt* and *flk* are also constitutively expressed in normal islet endothelium of control mice [27] (for technical reasons we have not been able to assess expression of the FGF receptors in the islet endothelium). Remarkably, the expression of these four genes encoding aFGF, VEGF and the VEGF receptors persists at similar levels in all stages of islet tumorigenesis in the RIP-Tag transgenic mice (Figure 3, see also [27]). Thus, there is no apparent modulation of gene expression for these two potent angiogenesis inducers that could explain the angiogenic switch in this model.

We suggest that these two models are revealing alternative facets of the angiogenic switch. The squamous cell model involves transformation of an epithelial cell type that is reasonably far removed from the capillary bed, which lies deep in the dermis, separated from the epidermis by a base-

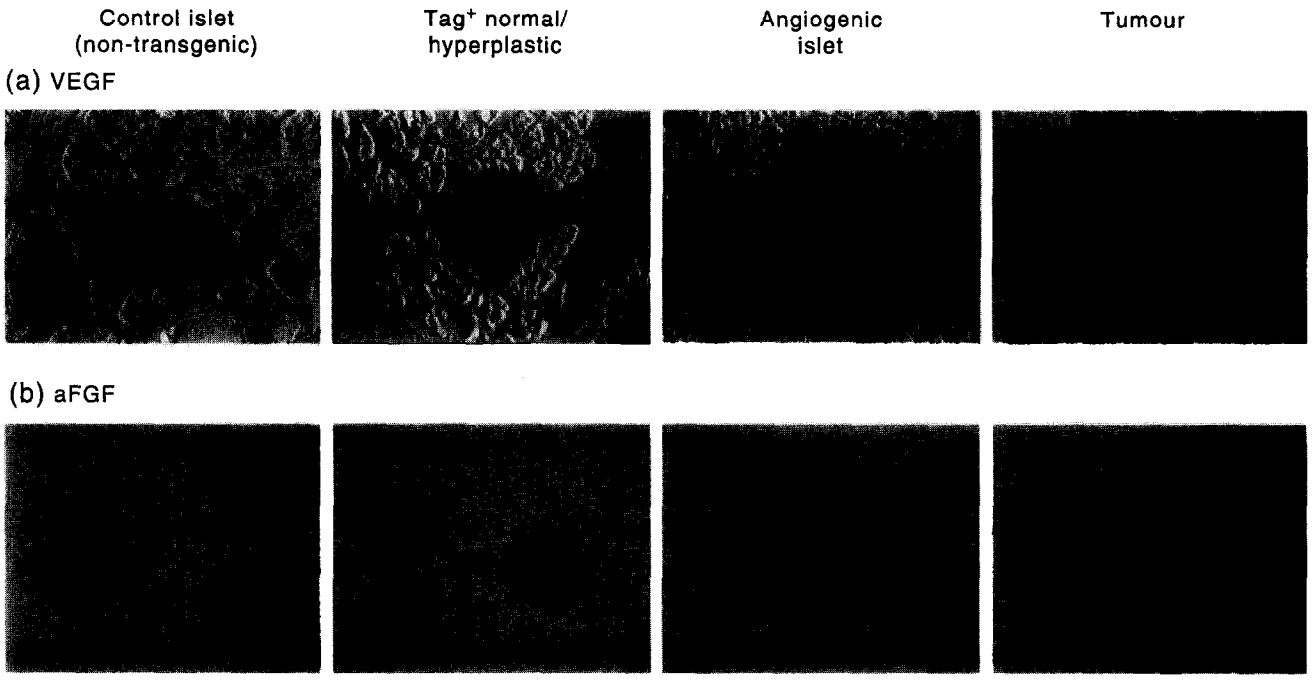

Figure 3. Both VEGF and aFGF are constitutively expressed in normal pancreatic islets and throughout islet cell carcinogenesis. Immunostaining of tissue sections from control and RIP-Tag2 transgenic mice was used to assess expression of VEGF (a) and aFGF (b) in the normal, pre-angiogenic and angiogenic stages of islet carcinogenesis. Representative islets at the various stages are shown, with the left column presenting control sections, and the others RIP1-Tag2 sections. The VEGF immunostaining utilised a polyclonal anti-human VEGF antiserum from Oncogene Sciences, Cambridge, Massachusetts while the aFGF analysis utilised affinity-purified rabbit anti-human FGF-1 antibodies provided by Dr Ralf Pettersson, Karolinska Institute, Stockholm, Sweden. In both cases, the primary antibody reaction was visualised with a secondary antibody conjugated with horseradish peroxidase (HRP) which was subsequently reacted with diaminobenzidine and nickel. The VEGF panels were photographed under Nomarski optics, whereas the aFGF panels used bright field illumination. Magnification: 50×.

ment membrane. Here, it would appear that VEGF and the FGFs (and perhaps other chemo-attractants) are upregulated so as to activate and recruit the distant vasculature, similar to classical bioassays in avascular tissues such as the cornea. In contrast, the pancreatic islets are extensively vascularised, to facilitate their functions of monitoring serum glucose levels and in consequence secreting insulin and other hormones to effect endocrine regulation of carbohydrate metabolism. In this case, aFGF, VEGF itself, as well as the newly recognised [28–31] VEGF-B and VEGF-C family members are all expressed in normal islet β cells [27] (G. Christofori, Research Institute of Molecular Pathology, Vienna, Austria), without activation of angiogenesis. We presume that the VEGFs in particular must serve a purpose distinct from angiogenesis for the quiescent islet vasculature; one likely function for the paracrine VEGF/VEGF-receptor circuit is in maintaining the endothelial fenestrae that characterise endocrine tissues [32]. Moreover, the islets may not be exceptional. The three non-allelic VEGF genes are collectively expressed at significant levels in many other endocrine and non-endocrine organs of the body [28–31], providing further support for the proposition that VEGF signaling serves other important roles for the homeostasis of normal tissue endothelium. Regarding the role of the VEGFs in activation of tumour angiogenesis, it should be noted that, in most of these tissues, the relative expression of the three VEGFs and their receptors in cognate tumours has not been rigorously compared; thus the demonstrable upregulation of VEGF, seen in hypoxic conditions [33–35] and in response to activated oncogenes, such as *H-RAS* [36], remains a potential component of the angiogenic switch. Nevertheless, the realisation that potent 'angiogenesis inducers' such as VEGF are constitutively expressed and may serve physiological functions for the endothelium in normal tissues suggests that changes in activator gene expression *per se* may not in all cases explain how vascular quiescence is maintained, and conversely, how angiogenesis is induced during tumorigenesis. The balance hypothesis for the angiogenic switch [20, 23] states that the endothelial cells integrate the effective levels of angiogenesis inducers and angiogenesis inhibitors in their micro-environment to govern their alternative cell differentiation states of quiescence or angiogenesis. Changes in the balance could, in principle, be affected by increasing activator gene expression, by altering the bioavailability or activity of the inducer proteins, for example, via processes such as post-translational modification or sequestration, or by reducing the effective concentrations of endogenous angiogenesis inhibitors, again via changes in gene expression or processing/bioavailability. The following two sections describe experiments which suggest that modification and bioavailability of angiogenesis activators, and the loss of angiogenesis suppressors, may each be general components of the angiogenic switch mechanism, in addition to regulation of activator gene expression.

MODIFICATION AND EXPORT OF a/bFGF: A NEW MODE OF REGULATION?

Both aFGF and bFGF are unusual growth factors in that they lack traditional signal sequences for secretion, implicat-

ing a novel mechanism of release from cells if they are to serve paracrine functions. Several studies have substantiated the notion that FGFs can be 'exported' out of cells by mechanisms that do not involve necrotic cell death and cell lysis. One experimental approach, by Maciag and colleagues, has demonstrated that 3T3 fibroblasts stably transfected with a aFGF expression vector release aFGF upon heat shock as a functionally inactive homodimer [22]. Similarly, we have shown that endogenous aFGF is released from the islet cell carcinomas discussed above. Again, the export pathway does not seem to involve either the traditional secretory pathway or cell lysis (G. Christofori, Research Institute of Molecular Pathology, Vienna, Austria). Interestingly, biochemical analysis has revealed novel characteristics: exported aFGF has reduced heparin affinity, and is released in high molecular weight forms of approximately 40 kDa (G. Christofori, Research Institute of Molecular Pathology, Vienna, Austria). In contrast, the 16 kDa monomer that binds heparin with high affinity remains cell associated. Another intriguing characteristic was the sequestration in the conditioned medium of the high molecular weight (HMW) aFGF forms, released both from the transfected 3T3 fibroblasts and from the β tumour cells; if conditioned medium was assayed using mitogenic activity assays or heparin affinity chromatography, no aFGF was detected. However, if the medium was first treated with high salt concentrations (1 M NaCl or ammonium sulphate precipitation), aFGF was recovered as HMW forms, as shown by mitogenic activity, heparin affinity (albeit reduced relative to recombinant aFGF), and antibody reactivity [22] (G. Christofori, Research Institute of Molecular Pathology, Vienna, Austria). The data suggest that the exported HMW forms of aFGF can be sequestered following export from the cell. Perhaps this sequestration reflects a capability of regulating this angiogenic inducer by storage, to be released locally as the tissue micro-environment dictates. The molecular basis of this sequestration, its possible manifestation as a new form of regulation, and the structure of the HMW forms of a FGF represent important topics for future study as does the additional question about whether the aFGF export capability is activated as part of the angiogenic switch in the islet cell tumorigenesis pathway. This issue is currently unresolved due to our inability to culture normal and pre-angiogenic pancreatic islet β cells in sufficient quantity for biochemical analysis. However, evidence for regulated export of the related angiogenesis factor bFGF has come from analysis of the BPV1.69 transgenic murine model of dermal fibrosarcoma.

In the multistage pathway to fibrosarcoma, the angiogenic switch occurs at the aggressive fibromatosis stage (Figure 1). When conditioned medium from cell lines derived from each stage of the pathway was tested for mitogenic activity on cultured capillary endothelial cells, only the lines derived from angiogenic lesions (aggressive fibromatosis and fibrosarcoma) showed mitogenic activity [16]. A survey revealed bFGF gene expression at similar levels in all cell lines, irrespective of the presence or absence of mitogenic activity in conditioned medium. Thus, changes in bFGF gene expression did not appear to provide an explanation for the observed angiogenic switch. In addition, VEGF is also expressed at similar levels throughout the pathway (G. Bergers, University of California, San Francisco, U.S.A.). However, striking differences were observed in the localisation of bFGF protein. In the pre-angiogenic normal and mild fibromatosis cell lines, bFGF remained cell associated, whereas immunoreactive bFGF was detected in medium conditioned by cell lines from angiogenic aggressive fibromatosis and fibrosarcoma. This observation led to the hypothesis that the angiogenic switch in this pathway involves the selective release of bFGF by cells in the angiogenic stages, but not by normal and pre-angiogenic stage cells.

The discovery of novel HMW forms of aFGF in the islet cell model led to an evaluation of the characteristics of the bFGF being released by the fibrosarcoma cells. Much like aFGF, the bFGF in the conditioned medium was found to be in HMW forms with reduced heparin affinity. Variable sequestration of bFGF in salt-sensitive complexes was also observed (G. Christofori, Research Institute of Molecular Pathology, Vienna, Austria). Therefore, in the two transgenic mouse models analysed, novel forms of an FGF lacking a traditional signal sequence for secretion are being exported by the tumour cells, consistent with their participation in the control of angiogenesis. Moreover, we have detected export of similar HMW forms of bFGF from several human breast cancer cell lines (G. Christofori, Research Institute of Molecular Pathology, Vienna, Austria). To summarise, we have detected three novel attributes of a/bFGF that appear pertinent to tumour angiogenesis: (i) the selective export out of transformed cells that were populating angiogenic lesions; (ii) sequestration of mitogenic activity once exported; and (iii) modifications that effect higher molecular weight forms with reduced heparin affinity. The common pattern of novel forms of exported a/bFGF shown by three distinct tumour cell types (mouse β islet, mouse fibroblast and human mammary epithelial) suggest that post-translational modification and selective export of a/bFGF may prove a common feature of the tumour phenotype. The export of a/bFGF by cancer cells may in part explain the markedly elevated levels of FGF frequently detected in the serum and urine from patients with a variety of malignancies (reviewed in [37, 38].

ANGIOGENESIS INHIBITORS AND SUPPRESSORS: EARLY CLUES IN TRANSGENIC MICE

It is becoming increasingly evident that endogenous endothelial inhibitors are a critical component for the regulation of angiogenesis, most likely equally important to the activators. As discussed above, the evidence increasingly supports a model by which endothelial activity is controlled by a changing balance of activators and inhibitors (reviewed in [20, 23]). Moreover, Bouck and colleagues have demonstrated that loss of tumour suppressor gene function can result in downmodulation of angiogenesis inhibitors and activation of the switch (reviewed in [23]), an example being the control of the inhibitor thrombospondin (TSP-1) by the wild-type p53 tumour suppressor protein [39]. Currently, angiogenesis inhibitors have not been directly implicated in the angiogenic switch during tumorigenesis in transgenic mouse models. However, the constitutive expression of angiogenesis activators in the islet cell model suggests counter-regulation of endothelial activity by inhibitors. While it is formally possible that sequestration of aFGF by normal

islet β cells might render the secreted VEGF insufficient to induce angiogenesis, it seems more likely that potent inhibitors must also be made by normal islets, to maintain vascular quiescence. As such, activation of the endothelium in angiogenic islets is predicted to be controlled by downregulation of inhibitor(s). A candidate for this putative angiogenic inhibitor is a genetic locus located on chromosome 16, (called *Loh2*) that is characteristically lost, beginning at the angiogenic islet stage, concomitant with the angiogenic switch [40]. A hypothesis would be that this suppressor gene, located on chromosome 16, controls expression of an angiogenesis inhibitor (much as p53 controls TSP-1), such that deletion of *Loh2* reduces expression and hence the local concentration of the angiogenesis inhibitor, shifting the balance to favour the constitutively expressed inducers, contributing to activation of the angiogenic switch. While merely a clue at this point, it seems likely that negative regulators of angiogenesis will prove to be involved in tumorigenesis, and accessible to investigation in transgenic models.

TRANSGENIC MODELS AS PLATFORMS FOR PRECLINICAL TESTING

Traditionally, the efficacy of antineoplastic therapies have been tested by administering test compounds to mice bearing subcutaneous tumours produced by inoculating syngeneic or immunodeficient mice with cultured tumour cell lines or primary tumour fragments. While informative, these bioassays are limited by the fact that the target is a tumour growing in an ectopic (unnatural) site, often following long-term cell culture. The advent of dozens of transgenic mouse models of tumorigenesis presents the opportunity to assess efficacy of anticancer compounds on a primary or metastatic tumour developing *de novo* in its natural tissue environment. Specifically, transgenic models present themselves as ideal platforms for preclinical testing of anti-angiogenesis drugs. In a recent prototypic example, RIP-Tag transgenic mice were treated with three angiogenesis inhibitors, AGM1470 (TNP470), alpha interferon, and minocycline [41]. Therapy began at 6 weeks of age at the onset of activation of the angiogenic switch. The regimen retarded angiogenesis, increased tumour cell apoptosis, and significantly reduced end-stage tumour volume to 10% of that of control mice (a 'T/C' of 0.1). This study suggests a number of experimental options to be explored in the future using the RIP-Tag mouse model, among which are: assessing additional angiogenesis inhibitors and alternative delivery modalities, including viral gene therapy approaches, and testing novel combinatorial effects of angiogenesis inhibitors, both with each other and with agents targeting other facets of the malignant phenotype, such as cell cycle control, telomerase or apoptosis.

Looking beyond our prototype model, an important focus of future investigation will be testing anti-angiogenesis inhibitors in transgenic models recapitulating human epithelial carcinogenesis. One example is the squamous cell carcinogenesis in the K14-HPV16 transgenic mice discussed above. In this model (Figure 1), invasive cancer of the epidermis arises in a pathway that resembles human squamous cancer. The time to malignant conversion is long, 9–12 months, and the incidence of cancers is low, 20% of affected mice [8]. In contrast, intense angiogenesis is characteristic of the dysplasias present in 100% of transgenic mice by 2–3 months of age. Thus, for models such as this, activation of angiogenesis can serve as a surrogate end-point biomarker [42] as well as a therapeutic target. Efficacious compounds could then be tested on established epidermal cancers and their metastases [18], either singly or in combinations. Recently, we have developed a derivative model of oestrogen-induced cervical carcinoma in these transgenic mice [43]. The cervical carcinomas develop through a multistage pathway in 80% of transgenic females after 6 months of oestrogen treatment, and thus could also be useful as a therapeutic platform. Similar logic may apply to other transgenic models of epithelial cancer, notably breast carcinoma development in transgenic mice with oncogenes regulated by the MMTV promoter [4]. While more lengthy and costly than traditional tumour transplant models, transgenic models may reveal more subtle but critical effects apparent only in a tumour developing in its natural tissue micro-environment. Indeed, the heterogeneity of the endothelium in different anatomical locations [44] suggests that there may be differential responses to therapeutic regimens in distinct locations.

CONCLUSION

This review has presented case studies of transgenic mouse technology and its application to questions and opportunities in tumour angiogenesis. Three diverse transgenic mouse models show a similar pattern of an angiogenic switch to persistent neovascularisation during tumour development, prior to the emergence of end-stage tumours. Histological evidence from three human cancers, of mammary epithelium, cervical epithelium, and cutaneous melanocyte, reveal angiogenic lesions that are arguably progenitors to invasive cancers. Thus, in terms of cancer biology, these transgenic models have revealed what may well prove to be a general parameter of multistage tumorigenesis, that of an angiogenic switch as a distinct, potentially rate-limiting step in the pathway to cancer. Following on from these observations, there are clear experimental avenues to investigate the mechanisms of the angiogenic switch, and to develop further transgenic models as platforms for preclinical testing of compounds that interfere with angiogenesis. Again, the prototype that exemplified these possibilities has been the RIP-Tag model of islet cell carcinoma, in which there is a discrete switch to the angiogenic phenotype among focal nodules at a reproducible age in mice that progress from normality to islet cell carcinoma in 12 weeks.

In the broader perspective, there are already more than 50 transgenic and gene knockout mouse models of cancer, with more on the horizon. Moreover, it can be envisaged that these technologies will be used in the future to develop increasingly accurate models for many of the major human cancers. In turn, there is clear reason to assess the angiogenic switch in these models; it seems likely that additional aspects of the switch mechanism will be revealed by such investigations. Since the balance of angiogenesis inhibitors and activators in different tissues and their tumours may well differ, so too may the mechanism of the switch. Thus, the biology may present distinct inroads into the design of specific interventions that block angiogenesis selectively and completely in a particular carcinogenesis pathway, raising

the prospects for clinical 'cures' in which angiogenesis inhibitors play a key role in the successful strategy.

1. Hanahan D. Dissecting multistep tumorigenesis in transgenic mice. *Annu Rev Genet* 1988, **22**, 479–519.
2. Hanahan D. Transgenic mice as probes into complex systems. *Science* 1989, **246**, 1265–1275.
3. Adams JM, Cory S. Transgenic models of tumor development. *Science* 1991, **254**, 1161–1167.
4. Frost P, Hart I, Kerbel RS. Cancer and metastasis reviews — transgenic mice. *Cancer Metast Rev* 1995, **14**, 77–161.
5. Hanahan D. Heritable formation of pancreatic β-cell tumors in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* 1985, **315**, 115–121.
6. Lacey M, Alpert S, Hanahan D. The bovine papillomavirus genome elicits skin tumours in transgenic mice. *Nature* 1986, **322**, 609–612.
7. Arbeit J, Munger K, Howley P, Hanahan D. Progressive squamous epithelial neoplasia in K14-HPV16 transgenic mice. *J Virol* 1994, **68**, 4358–4368.
8. Coussens LM, Hanahan D, Arbeit J. Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. *Am J Pathol*, in press.
9. Teitelman G, Alpert S, Hanahan D. Proliferation, senescence and neoplastic progression of β cells in hyperplastic pancreatic islets. *Cell* 1988, **52**, 97–105.
10. Christofori G, Naik P, Hanahan D. Insulin-like growth factor II is focally up-regulated and functionally involved as a co-factor in oncogene induced tumorigenesis. *Nature* 1994, **369**, 414–418.
11. Christofori G, Naik P, Hanahan D. Deregulation of both imprinted and expressed alleles of the IGF-II gene during β-cell tumorigenesis. *Nature Genet* 1995a, **10**, 196–201.
12. Naik PN, Karrim J, Hanahan D. Variation in apoptosis during multi-stage tumorigenesis: down-modulation contributes to tumor progression from angiogenic progenitors. *Genes & Dev*, 1996, **13**, 1847–1857.
13. Folkman J, Watson K, Ingber D, Hanahan D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* 1989, **339**, 58–61.
14. Sippola-Thiele M, Hanahan D, Howley PM. Cell heritable stages of tumor progression in transgenic mice harboring the bovine papillomavirus type 1 genome. *Mol Cell Biol* 1988, **9**, 925–934.
15. Bossy-Wetzel E, Bravo R, Hanahan D. Transcription factors JunB and c-Jun are selectively up-regulated and functionally implicated in fibrosarcoma development. *Genes & Dev* 1992, **6**, 2340–2351.
16. Kandel J, Bossy-Wetzel E, Radvany F, Klagsbrun M, Folkman J, Hanahan D. Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. *Cell* 1991, **66**, 1095–1104.
17. Hurlin PJ, Foley KP, Aver D, Eisenman RN, Hanahan D, Arbeit JM. Regulation of Myc and Mad during epidermal differentiation and HPV-associated tumorigenesis. *Oncogene* 1995, **11**, 2487–2501.
18. Arbeit JM. Transgenic models of epidermal neoplasia and multi-stage carcinogenesis. *Cancer Surveys* 1996, **26**, 7–34.
19. Rak JW, St Croix BD, Kerbel RS. Consequences of angiogenesis for tumor progression, metastasis and cancer therapy. *Anti-Cancer Drugs* 1995b, **6**, 3–18.
20. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996, **86**, 353–364.
21. Folkman J, Shing Y. Angiogenesis. *J Biol Chem* 1992, **267**, 10931–10934.
22. Friesel RE, Maciag T. Molecular mechanisms of angiogenesis: fibroblast growth factor signal. *FASEB J* 1995, **9**, 919–925.
23. Bouck N, Stellmach V, Hsu S. How tumors become angiogenic. *Adv Cancer Res*, in press.
24. Christofori G. The role of fibroblast growth factors in tumour progression and angiogenesis. In Bicknell R, Lewis CE, Ferrara N, eds. *Tumour Angiogenesis*. Oxford, Oxford University Press, in press.
25. Brown LF, Detmar M, Claffey K, Nagy JA, Feng D, Dvorak AM, Dvorak H. *Control of Angiogenesis*. Berlin, Birkhauser Verlag, 1996.
26. Arbeit JM, Olson D, Hanahan D. Upregulation of fibroblast growth factors and their receptors during multistage epidermal carcinogenesis in K14-HPV16 transgenic mice. *Oncogene* 1996, **13**, 1847–1857.
27. Christofori G, Naik P, Hanahan D. Vascular endothelial growth factor and its receptors, flt-1 and flk-1, are expressed in normal pancreatic islets and throughout islet cell tumorigenesis. *Mol Endocrinol* 1995, **9**, 1760–1770.
28. Oloffsson B, Pajusola K, Kaipainen A, *et al.* Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci USA* 1996, **93**, 2576–2581.
29. Joukov V, Pajusola K, Kaipainen A, *et al.* A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 1996, **15**, 290–298.
30. Lee J, Gray A, Yuan J, Luoh S-M, Avraham H, Wood WI. Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. *Proc Natl Acad Sci USA* 1996, **93**, 1988–1992.
31. Grimmond S, Lagercrantz J, Drinkwater C, *et al.* Cloning and characterization of a novel human gene related to vascular endothelial growth factor. *Genome Res* 1996, **6**, 124–131.
32. Roberts WG, Palade GE. Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *J Cell Sci* 1995, **108**, 2369–2379.
33. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 1992, **359**, 843–845.
34. Shweiki D, Neeman M, Itin A, Keshet E. Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: implications for tumor angiogenesis. *Proc Natl Acad Sci USA* 1995, **92**, 768–772.
35. Stein I, Neeman M, Shweiki D, Itin A, Keshet E. Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes. *Mol Cell Biol* 1995, **15**, 5363–5368.
36. Rak J, Mitsuhashi Y, Bayko L, *et al.* Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res* 1995a, **55**, 4575–4580.
37. Folkman J. Tumor angiogenesis. In Mendelsohn J, Howley PM, Israel MA, Liotta LA, eds. *The Molecular Basis of Cancer*. Philadelphia, W.B. Saunders Company, 1995, 206–232.
38. Folkman J. Clinical applications of research on angiogenesis. *N Engl J Med* 1995, **333**, 1757–1763.
39. Dameron KM, Volpert OV, Tainsky MA, Bouck N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* 1994, **265**, 1582–1584.
40. Parangi S, Dietrich W, Christofori G, Lander E, Hanahan D. Tumor suppressor loci on mouse chromosomes 9 and 16 are lost at distinct stages of tumorigenesis in a transgenic mouse model of islet cell carcinoma. *Cancer Res* 1995, **55**, 6071–6076.
41. Parangi S, O'Reilly M, Christofori G, *et al.* Antiangiogenic therapy of transgenic mice impairs de novo tumor growth. *Proc Natl Acad Sci USA* 1996, **93**, 2002–2007.
42. Boone CW, Kelloff GJ. Intraepithelial neoplasia, surrogate endpoint biomarkers and cancer chemoprevention. *J Cell Biochem* 1993, **17F** (Suppl.), 37–48.
43. Arbeit J, Howley P, Hanahan D. Chronic estrogen-induced cervical and vaginal squamous carcinogenesis in HPV16 transgenic mice. *Proc Natl Acad Sci USA* 1996, **93**, 2903–2935.
44. Dellian M, Witwer BP, Salehi HA, Yuan F, Jain RK. Quantitation and physiological characterization of angiogenic vessels in mice: effect of basic fibroblast growth factor, vascular endothelial growth factor/vascular permeability factor, and host microenvironment. *Am J Pathol* 1996, **149**, 59–71.

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