Acquired Expression of Periostin by Human Breast Cancers Promotes Tumor Angiogenesis through Up-Regulation of Vascular Endothelial Growth Factor Receptor 2 Expression

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The late stages of human breast cancer development are poorly understood complex processes associated with the expression of genes by cancers that promote specific tumorigenic activities, such as angiogenesis. Here, we describe the identification of periostin as a mesenchyme-specific gene whose acquired expression by human breast cancers leads to a significant enhancement in tumor progression and angiogenesis. Undetectable in normal human breast tissues, periostin was found to be overexpressed by the vast majority of human primary breast cancers examined. Tumor cell lines engineered to overexpress periostin showed a phenotype of accelerated growth and angiogenesis as xenografts in immuno compromised animals. The underlying mechanism of periostin-mediated induction of angiogenesis was found to derive in part from the up-regulation of the vascular endothelial growth factor receptor Flk-1/KDR by endothelial cells through an integrin αvβ3-focal adhesion kinase-mediated signaling pathway. These findings demonstrate the presence of a novel mechanism by which tumor angiogenesis is acquired with the expression of a mesenchyme-specific gene as a crucial step in late stages of tumorigenesis.

The development of human cancers is a multistep complex process by which cancer cells acquire the ability to overcome the restraints imposed by the surrounding normal tissue microenvironment (7). This process is believed to be driven by the intrinsic genomic instability of cancer cells to express genes that confer selective advantages under the adverse growth conditions associated with a rapidly expanding tumor mass, such as hypoxia and a poor supply of nutrients. After reaching a critical mass, cancer cells have to find ways to promote angiogenesis in order to progress and expand during late stages of tumorigenesis (4, 5). To this end, a number of genes, such as the vascular endothelial growth factor (VEGF), have been demonstrated to play critical roles in the development of tumor vasculature (4, 5, 10). However, much more remains to be learned about the molecular nature of still unidentified players and their modes of action in promoting tumor angiogenesis.

Recently, large-scale efforts have been made to determine gene expression pattern differences between various types of human cancers and their corresponding normal tissues by using the serial analysis of gene expression (SAGE) and gene array analyses (14, 33–35, 37). Indeed, significant differences in gene expression patterns have been revealed by these studies. In breast cancer, for example, such investigations have led to the application of gene array analysis in the diagnosis, prognosis, and design of rational treatment of patients according to the molecular signatures of the individual tumors (21, 22, 32, 35). In the meantime, although the alterations of oncogenes and tumor suppressor genes have shown a close association with the progression of human cancers based on their defined functions, less is known about the specific contributions of a large number of genes whose expression patterns are also significantly changed during the tumorigenic process. Particularly interesting is the observation that mesenchyme-specific genes, normally associated with osteoblasts, are highly expressed by various types of human cancers (17, 31). However, the expression of mesenchyme-specific genes has not been functionally linked to the development of specific tumor phenotypes.

To address this question, we sought to determine the potential contributions of such candidate genes to specific phenotypic changes associated with the progression of late-stage tumorigenesis and identified a mesenchyme-specific gene product, periostin, as a novel angiogenic factor whose overexpression by human breast cancers leads to the significant enhancement of angiogenesis. The angiogenic activity of periostin correlated with the increased expression of the VEGF receptor Flk-1/KDR by endothelial cells through an integrin αvβ3-focal adhesion kinase (FAK)-mediated signaling pathway. These findings indicate that epithelial cell-derived tumors may gain the capabilities to generate more blood vessels, invade, and metastasize during late stages of tumorigenesis by the acquired expression of genes whose functions are normally associated only with mesenchymal cells.

MATERIALS AND METHODS

Gene array analysis. Total RNA from 50 primary breast cancers and three normal primary mammary epithelial cultures was labeled and hybridized to Affymetrix Gene Chips (FL arrays and 6,800 genes). Data were expressed as the average differences between the perfect match and mismatch probes for the periostin gene (see http://data.cgt.duke.edu for the raw gene array data).

Generation of periostin-producing cells. Full-length human periostin cDNA (MluI-XhoI) was subcloned into a retroviral pCMV-neo-vector. 293T retroviral packaging cells were transfected with the periostin construct or vector control in

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the presence of pCL 10A1 vector by using Fugene 6 as the delivery vehicle. Forty-eight hours after transfection, the supernatant was harvested and filtered through a 0.4-

m-pore-size filter, and the virus-containing medium was used to infect cells. Selection with 800 g of G418/ml was started 48 h after infection. For 293T, B16F1, and MDA-MB-231 cell lines, the drug-resistant cell populations were used for subsequent studies of tumor formation. For MCF-7 cells, a single stable clone that expresses periostin was isolated.

Tumor xenograft analysis in mice. Four-week-old female SCID-Beige mice (Charles River, Wilmington, Mass.) were subcutaneously injected with control or periostin-producing 293T (2.5 \times 10^7), MDA-MB-231 (1 \times 10^7), or B16F1 (0.4 \times 10^7) cells in 0.2 ml of Hank's balanced buffer without calcium and magnesium. The growth of solid tumors from the injected cells was monitored daily for up to 2 or 4 weeks before the animals were sacrificed to remove tumors for analysis. For testing the effect of Flk-1/KDR inhibitor SU5416 on tumor growth, mice were injected subcutaneously to the opposite flank of the tumor cell injection with SU5416 (20 mg/kg) in suspension of a diluent (0.5% carboxymethylcellulose sodium, 0.9% sodium chloride, 0.4% polysorbate 80, 0.9% benzyl alcohol) every other day for 5 weeks. Control mice were treated with the diluent alone. The removed tumors were measured and calculated as follows: volume = length \times width^2 \times 0.52.

Hemoglobin content measurement. Tumor tissue (0.25 g) was removed immediately after animal sacrifice, and blood was mechanically extracted in phosphate-buffered saline containing heparin. Hemoglobin concentration was determined as previously described (2).
Immunohistochemical analysis. For frozen tissue samples, tumor slides (thickness, 6 to 10 μm) were fixed in 2% paraformaldehyde. Sections were stained for the presence of CD31 (Becton Dickinson Labware, Bedford, Mass.), Flk-1/KDR (Research Santa Cruz, Santa Cruz, Calif.), or periostin according to the manufacturer’s instructions (Vector Laboratories, Burlingame, Calif.).

Generation of recombinant periostin. Full-length human periostin cDNA with a His tag was subcloned into pFastBac1 vector (Life Technologies, Rockville, Md.). Following transformation and amplification in Escherichia coli DH10Bac, bacmid DNA containing periostin was transfected into Sf9 insect cells (Invitrogen, Carlsbad, Calif.) by using Cellfectin reagent (Life Technologies), and baculoviral medium was produced. Recombinant periostin was generated 48 h after infection of High-5 cells with viral medium. A Ni-nitrilotriacetic acid column was used to purify recombinant periostin according to the manufacturer’s instructions (Life Technologies), and pure periostin was finally produced through a PD-10 column (Amersham Pharmacia Biotech, Piscataway, N.J.).

Cell migration assay. Human microvascular endothelial cells (HMVEC) (2 x 10⁵) were preincubated with serum-free medium for 12 h and transferred onto transwells (24-well plates) for migration assays as previously described (30).

[³H]thymidine incorporation assay. HMVEC were grown in 12-well plates to subconfluence, and the culture medium was changed to conditioned medium from the parental, control, and periostin-producing MCF-7 cells for 12 h. After washing, VEGF (10 ng/ml) was added for 12 h. A total of 2 μCi of [³H]thymidine was then added to each well for 6 h. After being thoroughly washed with phosphate-buffered saline, the cells were scraped and precipitated with 200 μl of 10% trichloroacetic acid. ³H radioactivity was solubilized in 0.3 M NaOH and quantitated by liquid scintillation count.

Western blot analysis. Serum-free media from cultured confluent cells were collected, and the presence of secreted periostin was determined by immunoblotting with a polyclonal antiperiostin antibody. The antibody was generated by immunizing the rabbits with recombinant periostin protein and was purified through an affinity column. For the measurement of Flk-1/KDR and FAK activation, an anti-phospho-Tyr 951 antibody (Research Santa Cruz) and an anti-phospho-Tyr 681 FAK antibody (Biosource, Camarillo, Calif.) were used to detect phosphorylated Flk-1/KDR or FAK in cell lysates.

RESULTS
Identification of periostin as a mesenchymal gene overexpressed in human cancers. To understand the nature of genes
that are responsible for the pathological progression associated with late stages of human cancer development, we searched the database derived from recently published results in the SAGE library in order to identify candidate genes that are highly expressed in various types of human cancers in comparison to normal tissue (14, 33, 34, 37). Among several candidates we found one gene, periostin (also termed OSF-2 for osteoblast-specific factor-2), that was originally defined as a secreted factor associated with osteoblastic cell function during bone development (8, 14, 29) and that was overexpressed in a broad range of human cancer types, including breast cancer (6, 9, 25, 26). To establish further a more comprehensive profile for periostin expression in human breast cancer, we took advantage of the available data from gene expression array analyses generated from both normal breast tissues and primary breast tumor samples from patients (35). Consistent with previously reported findings that periostin is specifically expressed by osteoblasts, the level of periostin expression was undetectable in normal breast tissues or in an immortalized cell line derived from normal mammary epithelial cells (Fig. 1A). However, the expression of periostin was readily detected in the vast majority of breast tumor samples, with an average level of periostin expression 20-fold higher than the baseline as defined by the value of gene array data obtained from normal breast tissues (Fig. 1A). As shown in Fig. 1B, 86% of the tumor samples overexpressed periostin at levels fivefold higher than the baseline. Among the samples, 11 (20%) had extremely high levels of periostin mRNA expression (>30-fold over baseline).

To determine if the higher levels of periostin mRNA expression revealed by gene array analysis were directly linked to increased levels of periostin protein expression, we performed Western blot analysis with protein extracts from several tumor samples. As shown in Fig. 1C, both an unprocessed and a processed shorter form of periostin were found to be highly expressed in tumor tissues from three different patients, whereas periostin was absent from normal breast tissue. Breast tumor tissues are known to contain both epithelial cancer cells and stromal cells. To determine the distribution of periostin within breast cancer tissues, we performed an immunohistochemical analysis by using a specific antiperiostin antibody. As shown in Fig. 1D, extensive staining of periostin was found mainly in areas containing carcinoma cells within the tumor tissue sections. The source of periostin production appeared to be the epithelial cancer cells, based on the results of RNA in

FIG. 2—Continued.
we measured the content of hemoglobin as a reappearance of higher levels of hemorrhage compared to levels in tumor mass derived from periostin-producing cells had an appearance of a more intense staining of the marker CD31 (Fig. 2E and F). This result firmly established that the presence of periostin is intimately associated with the presence of a higher density of vasculature and endothelial cells in tumors grown as xenografts. Taken together, our data strongly suggest that periostin may act to promote tumor growth by inducing tumor angiogenesis.

Overexpression of periostin is associated with enhanced tumor growth and angiogenesis. The acquired expression of periostin by various types of cancers including breast cancer suggests that periostin may be intimately associated with the progression of tumor development. To test this hypothesis, tumor cells were engineered to produce periostin and injected into immunocompromised animals, and the growth characteristics of the resulting solid tumors were examined. Specifically, we used three cell lines that do not express endogenous periostin at a detectable level: the 293T cell line derived from human kidney epithelial cells, the highly invasive mouse melanoma cell line B16F1, and the metastatic human breast cancer line MDA-MB-231. After introducing the periostin gene into these cells by means of a retroviral vector infection system, we examined the expression of periostin by performing Western blot analysis on the conditioned media harvested from the stably infected cell populations.

As shown in Fig. 2A, all three cell populations, compared to control vector-infected cells, secreted significant amounts of periostin to the media. Interestingly, the proliferation rate of the periostin-producing cells was found to be noticeably slower than that of the control cells in culture (data not shown), suggesting that periostin does not confer a proliferation-promoting effect on tumor cells in vitro. These cell populations were then injected subcutaneously into SCID-Beige mice, and the growth characteristics of resulting tumors were analyzed over a period of 14 or 28 days. For 293T cells, the volume of tumors derived from periostin-producing cells was two- to fourfold higher than that of tumors from control cells on day 28 (Fig. 2B). Strikingly, expression of periostin also enhanced the growth of tumors derived from the B16F1 and MDA-MB-231 cells (Fig. 2B), despite the fact that these cell lines are known to be among the most aggressive types in tumor formation when they are grown as xenografts. Interestingly, we found that the growth of tumors derived from the vector-transfected control cells was not affected by the presence of periostin-producing tumors in the same animal when the two cell populations were injected into opposite flanks (data not shown), suggesting that periostin exerts its effect locally rather than systematically.

In the process of dissecting the tumors, we noticed that the tumor mass derived from periostin-producing cells had an appearance of higher levels of hemorrhage compared to levels in tumors from control cells (Fig. 2C). Based on this observation, we measured the content of hemoglobin as a reflection of the amount of blood contained within the tumors. As shown in Fig. 2D, the hemoglobin content in tumors derived from periostin-producing cells was on average 30% higher than that in tumors derived from control cells, suggesting a possible difference in the density of blood vessels between the two forms of tumors.

To explore further the molecular mechanism of periostin-induced angiogenesis, we examined whether periostin could have a direct effect on the regulation of expression or activity of certain angiogenic factors and their receptors that are known to play important roles in tumor angiogenesis. VEGF and its receptor Flk-1/KDR have been extensively documented to be involved in the induction of angiogenesis during the development of solid tumors (13, 19, 28, 36). VEGF secreted from tumor cells, as well as stromal cells, exerts its angiogenic effects on endothelial cells by the activation of Flk-1/KDR. We found by Western blot analysis that the level of VEGF in conditioned medium from periostin-producing MCF-7 cells was no different from the amount in medium derived from control cells (data not shown). In addition, treatment of parental MCF-7 cells that lack the expression of endogenous periostin with recombinant periostin produced in a baculovirus system did not enhance VEGF production (data not shown).
In contrast, incubation of HMVEC with recombinant periostin or the conditioned medium from periostin-producing MCF-7 cells resulted in up-regulation of Flk-1/KDR expression in a dose- and time-dependent manner (Fig. 4A and B). Consistent with the results shown in Fig. 2E, immunostaining of tumor sections with the anti-Flk-1/KDR antibody also confirmed the presence of a higher level of presence of this VEGF receptor associated with the higher density of blood vessels in tumors derived from periostin-producing cells (Fig. 4C). To determine if up-regulation of Flk-1/KDR expression leads to an increase in the sensitivity of endothelial cells to VEGF, we pretreated HMVEC with recombinant periostin and subsequently measured the cellular proliferative response to VEGF. As shown in Fig. 4D, the proliferation of HMVEC was increased in response to VEGF in comparison to untreated control cells, and this stimulatory response was further potentiated by the periostin pretreatment. To confirm that the significant enhancement in response to VEGF by the periostin-pretreated HMVEC was the result of the increased activity of Flk-1/KDR due to its up-regulated expression, we examined the potential changes in the kinase autophosphorylation activity of Flk-1/KDR by using a specific anti-phospho-Tyr 951 antibody (3). As shown in Fig. 4E, periostin pretreatment of HMVEC led to a significant increase in the amount of phosphorylated Flk-1/KDR as a direct reflection of the increase in Flk-1/KDR expression and, consequently, enhancement in its activation in response to VEGF.

A recent report suggested that periostin could functionally interact with integrins to mediate the adhesion and migration of human ovarian carcinoma cells (6). To test the possibility that periostin may induce Flk-1/KDR expression through interaction with integrins in endothelial cells, we examined the profile of integrin expression in HMVEC and found those cells to express predominantly αvβ3 integrins (data not shown). Treatment of HMVEC with recombinant periostin did not alter the expression profile of the integrins (data not shown). We next probed if interference with the function of integrins by specific anti-integrin antibodies has an effect on the ability of periostin to mediate cell adhesion and induction of Flk-1/KDR in HMVEC. As shown in Fig. 5A, treatment of HMVEC with a specific anti-αvβ3 integrin antibody inhibited adhesion of these cells to the culture wells precoated with periostin. Consistent with this result, treatment of HMVEC with periostin in the presence of the anti-αvβ3 integrin antibody prevented the
The induction of Flk-1/KDR (Fig. 5B). The specificity of the blockade of periostin activity achieved by interfering with the function of αβ3 integrin was demonstrated by the lack of an effect on the periostin-mediated cell adhesion and induction of Flk-1/KDR expression when a specific anti-αβ3 integrin antibody was used in the same assays (Fig. 5). The initial step of integrin signaling involves the activation of FAK. Consistent with this notion, we found that transient stimulation of HMVEC with periostin augmented the phosphorylation of FAK on tyrosine 681 (Fig. 5B), an event indicative of the activation of FAK (1). The increase in FAK phosphorylation on Tyr 681 was reversed to the basal level by the presence of the anti-αβ3 integrin antibody but not the anti-αβ5 integrin antibody. Taken together, these results strongly suggest that the αβ3 integrin-FAK signaling pathway plays an essential role in mediating the effect of periostin on the up-regulation of Flk-1/KDR expression in HMVEC.

Inhibition of VEGF receptor abolished periostin-induced enhancement in tumor growth and angiogenesis. Finally, to establish a firm role for Flk-1/KDR up-regulation in the mediation of the proangiogenic activity of periostin, we employed two specific inhibitors of Flk-1/KDR in our functional assays. As shown in Fig. 6A, we found that the periostin-induced increases in cellular migration by the HMVEC were significantly inhibited by the presence of two inhibitors, SU5416, a compound that has been previously reported to specifically block the kinase activity of Flk-1/KDR (12), and sFlk, the soluble form of a VEGF receptor that has been demonstrated to sequester VEGF (16). Importantly, the increased tumor growth that resulted from the production of periostin was completely reversed by the presence of the inhibitor SU5416 in our xenograft model system (Fig. 6B). This decrease in tumor growth correlated directly with a reduction in angiogenic activity within the tumor mass derived from animals treated with the inhibitor SU5416, since the extent of vasculature density in those tumors was reduced to a level similar to that detected in control tumor sections (Fig. 6C and D).

Hence, periostin acts to increase the expression of the VEGF receptor (Flk-1/KDR), which in turn renders the cells more sensitive to the action of VEGF as demonstrated by a significant increase in both biological and biochemical responses by the HMVEC. Most importantly, the increased activity of the VEGF receptor is directly linked to the increased growth of tumor xenografts as a consequence of periostin production. Taken together, these data strongly support the notion that the up-regulation of Flk-1/KDR expression and, consequently, the sensitization of endothelial cells to the potent angiogenic factor VEGF are at least partially responsible for periostin-mediated tumor angiogenesis.

**DISCUSSION**

The present results reveal a novel mechanism by which angiogenesis is promoted in tumor progression in vivo. In this case, the acquired expression of periostin, an osteoblast-specific secreted protein known to be associated with cell adhesion activities for bone formation and development, by the epithelial cell-derived tumors leads to a significant enhancement in angiogenesis and tumor progression. Periostin produced by the breast carcinoma cells within the tumor mass acts in a para-
crine manner to enhance the responsiveness of microvessel endothelial cells that have been recruited into the tumor and coopted to generate the neovascularization associated with the rapid expansion of the tumor mass and possibly subsequent metastasis. In fact, another study has found that overexpression of periostin is associated with the increased metastasis of colon cancers (S. Bao, G. Ouyang, X. Bai, Z. Huang, C. Ma, M. Liu, R. Shao, R. M. Anderson, J. N. Rich, and X.-F. Wang, submitted for publication). Mechanistically, the up-regulation of expression of a critical receptor for the potent angiogenic factor VEGF represents a novel signaling pathway through which tumors can act to promote the proliferation, migration, and vessel formation activities of endothelial cells.

Importantly, the acquired expression of periostin by tumors of epithelial origin described here is certainly not an isolated and rare event during tumorigenesis, since a number of other examples of acquired expression of mesenchymal genes by epithelial-cell-derived tumors can be found in the literature (17, 18, 23). For example, osteonectin, also termed SPARC, whose physiological activity is believed to be associated mainly with osteoblast function, has been found to be overexpressed by a wide range of human cancer types (15, 24, 31). As a secreted polypeptide without sequence homology with periostin, osteonectin has been implicated to promote tumor progression and angiogenesis (10). Thus, the common feature for this group of structurally unrelated and functionally diverse molecules, either as secreted or extracellular matrix-associated proteins, is that their physiological functions are normally associated strictly with cells that derive from mesenchymal origins such as osteoblasts. By acquiring the expression of such mesenchymal genes, the epithelial carcinoma cells gain the abilities that are normally associated with mesenchymal cells, an event which appears to correlate with the progression into a more aggressive cancer phenotype.

Our immunohistochemical staining data suggest that periostin is present predominantly in areas containing cancer cells within the tumor mass. Based on the result of RNA in situ hybridization, the source of periostin production was determined to be the carcinoma cells, a notion that is consistent with the conclusion of a recent study on the production of periostin by human ovarian cancer cells (6). However, another recent report suggested that the localization of periostin mRNA was mainly associated with the stromal portion of the tumor tissue sample isolated from a breast cancer patient (27). In any event, periostin produced by either cell type within the tumor tissue could exert a similar paracrine effect on the endothelial cells recruited to the tumor mass to promote angiogenesis. As a related matter, we have also observed that the tumor cells engineered to produce periostin in culture containing a normal level of fetal bovine serum had a growth disadvantage in comparison to control cells (data not shown). In fact, we had to resort to the use of tumor cell populations in which ectopic periostin expression was engineered by retroviral infection and transient drug selection since we could not obtain multiple lines of stable clones that overexpress periostin; the exception was a single stable clone from MCF7 cells which was used as a source of periostin production in a number of the experiments described above. Thus, although periostin may confer an advantage for the growth of breast tumors in vivo by altering the microenvironment through the induction of angiogenesis, its overexpression may impose a growth disadvantage when the tumor cells are grown in culture.

These observations reveal the vital importance of relying on evidence derived from primary human cancer samples rather than established cell lines to draw major conclusions on the mechanism and involvement of specific genes in tumorigenesis. Furthermore, the tumor-promoting effect of molecules such as periostin can only be revealed by in vivo analysis in animal models, rather than solely by in vitro studies in cell culture. The normal functions of this type of gene are often not associated...
FIG. 6. Inhibition of Flk-1/KDR abolished periostin-induced enhancement in tumor growth and angiogenesis. (A) Inhibition of Flk-1/KDR blocks periostin-induced cell migration. HMVEC were employed for a migration assay in the presence of SU5416 (20 μM), sFlk-1 (100 ng/ml), periostin (100 ng/ml), or different combinations as indicated. *P ≤ 0.05 compared with the group treated with periostin alone. (B) The enhanced tumor growth by periostin-producing 293T cells was completely reversed by the Flk-1/KDR inhibitor SU5416. The time course of 293T tumor growth as measured by tumor volume was plotted with control, periostin-producing cells, or periostin plus SU5416 as described in Materials and Methods. *P ≤ 0.05 compared with the group treated with periostin alone. The enhanced tumor growth by periostin-producing 293T cells was completely reversed by the Flk-1/KDR inhibitor SU5416 compared with the group treated with periostin alone. (C and D) Reduced tumor growth in the presence of SU5416 was correlated with a reduction in angiogenesis. Hemoglobin content in tumors was measured as previously described. *P ≤ 0.05 compared with control; †P ≤ 0.05 compared with periostin. Tumor sections were immunohistochemically stained with anti-CD31 and Flk-1/KDR antibodies, and representative sections of each type of sample are shown (magnification ×200).
with the promotion of cell proliferation, in contrast to the roles of many defined oncogenes. Instead, this group of proteins may exert their influence on tumorigenesis by changing the microenvironment of tumor growth through the regulation or alteration of cell adhesion, composition of the extracellular matrix, and the activities of stromal cells within and surrounding the tumor mass.

Based on the results of this study with periostin as an example, we suspect that different types of human cancers derived from epithelial origins, and even cancers from the same tissue type but derived from different individual patients, may acquire the expression of different sets of mesenchymal-specific genes to gain different mesenchymal-associated capabilities during late stages of tumorigenesis. In other words, the heterogeneity in the functions of this group of genes may confer different tumorigenic capabilities on the cancer cells that acquire the expression of such genes, creating another layer of heterogeneity and complexity for each type and even each case of cancer development. Thus, identification and characterization of such genes will become crucial for a full understanding of the molecular events associated with late stages of tumorigenesis, particularly angiogenesis and metastasis, and for the future development of specific and effective regimens for a cancer treatment tailored to each individual patient. To this end, the availability of a vast amount of data on gene expression profiles derived from the SAGE library and gene array analyses of a broad range of human cancer types in comparison to their counterparts of normal tissue has provided us with a golden opportunity through functional genomics to search for and identify candidate genes that fit this profile. Since the activities of these molecules may not be associated with the promotion of cell proliferation, the foremost criterion for evaluating the potential contribution of any candidate genes to the progression of tumorigenesis will have to be based on an assessment of the ability of those genes to promote tumorigenesis in vivo studies of xenografts or transgenic animal model systems.

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