Transcriptional Switch of Dormant Tumors to Fast-Growing Angiogenic Phenotype

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Abstract

Tumor dormancy has important implications for early detection and treatment of cancer. Lack of experimental models and limited clinical accessibility constitute major obstacles to the molecular characterization of dormant tumors. We have developed models in which human tumors remain dormant for a prolonged period of time (>120 days) until they switch to rapid growth and become strongly angiogenic. These angiogenic tumors retain their ability to grow fast once injected in new mice. We hypothesized that dormant tumors undergo a stable genetic reprogramming during their switch to the fast-growing phenotype. Genome-wide transcriptional analysis was done to dissect the molecular mechanisms underlying the switch of dormant breast carcinoma, glioblastoma, osteosarcoma, and liposarcoma tumors. A consensus expression signature distinguishing all four dormant versus switched fast-growing tumors was generated. In alignment with our phenotypic observation, the angiogenic process was the most significantly affected functional gene category. The switch of dormant tumors was associated with down-regulation of angiogenesis inhibitor thrombospondin and decreased sensitivity of angiogenic tumors to angiostatin. The conversion of dormant tumors to exponentially growing tumors was also correlated with regulation and activation of pathways not hitherto linked to tumor dormancy process, such as endothelial cell–specific molecule-1, s-ecto-nucleotidase, tissue inhibitor of metalloproteinase-3, epidermal growth factor receptor, insulin-like growth factor receptor, and phosphatidylinositol 3-kinase signaling. Further, novel dormancy-specific biomarkers such as H2BK and Eph receptor A5 (EphA5) were discovered. EphA5 plasma levels in mice and mRNA levels in tumor specimens of glioma patients correlated with diseases stage. These data will be instrumental in identifying novel early cancer biomarkers and could provide a rationale for development of dormancy-promoting tumor therapy strategies. [Cancer Res 2009;69(3):836–44]

Introduction

It is widely accepted that human tumors can arise in the absence of angiogenic activity and exist in a microscopic dormant state for months to years without neovascularization (1). The disease stage of cancer, therefore, seems to be a late event in tumor development. Dormant tumors are defined as microscopic (diameter of ~1 mm) and asymptomatic cancerous lesions that remain occult for prolonged periods of time. They represent the earliest stages in tumor development and are highly prevalent in humans (2). Moreover, tumor dormancy is also attributed to the long latency periods frequently observed in cancer patients between the primary diagnosis and treatment and the potential clinical evidence of local recurrence or distant metastasis. Based on tumor type and stage, the dormancy period may range from years to even decades between the initial therapy and the occurrence of relapsed tumors or recurrent metastatic disease (3).

Although the clinical implication of tumor dormancy in prevention and treatment of tumors has intrigued the medical community for years, there is a paucity of molecular markers and mechanistic understanding. A critical limitation confronting the field of tumor dormancy is the lack of suitable experimental models as well as consistent and abundant sources of dormant tumor cells. Although several mechanisms have been proposed to affect tumor dormancy (1, 4–10), it is still unclear what keeps these tumors in a microscopic size and prevents their expansion.

We have previously established in vivo models of human breast cancer, glioblastoma, osteosarcoma, and dormant liposarcoma in immunocompromised [severe combined immunodeficient (SCID) mice; refs. 8, 11]. Using these models, we have shown that dormant microscopic tumors reside in mice for a long period (>90 days) until they switch to become fast-growing angiogenic tumors. In this work, we aimed to characterize the consensus molecular fingerprint of tumor dormancy using genome-wide expression profiling.

We found angiogenesis-related genes to be the most significantly enriched functional category among the differentially regulated genes. The endogenous angiogenesis inhibitor thrombospondin, as well as angiostatin and endostatin binding proteins (angiomotin and tropomyosin, respectively), were found to be up-regulated in all dormant tumor cell lines examined. Of note, we found that angiostatin selectively binds and inhibits tumor migration in angiomotin-expressing dormant tumor cells. Furthermore, we found elevated RNA levels of key cancer pathways in angiogenic fast-growing tumors, including epithelial growth factor receptor (EGFR)-I, insulin-like growth factor type I receptor (IGF-IR), and phosphatidylinositol 3-kinase (PI3K). Importantly, Eph receptor A5 (EphA5) levels in plasma of mice bearing occult, dormant glioma was significantly higher than in control mice. It also correlated with disease stage in cancer patients.

The consensus dormancy signature reported here may suggest molecular instructions to address the unmet medical need of
discovering novel early tumor dormancy biomarkers. Elucidating the molecular mechanisms underlying human tumor dormancy may also provide an attractive venue for rational design of treatment strategies aiming to either prevent or reverse the switch of dormant tumors to the angiogenic fast-growing phenotype.

Materials and Methods

Cell lines, tissue culture, and surgical specimens. Human breast adenocarcinoma (MDA-MB-436), osteosarcoma (KHSO-240S), glioblastoma (T98G), and liposarcoma (SW1783) cell lines were obtained from American Type Culture Collection. All cell lines were maintained as previously described (8, 11). Migration assays were done as previously described (12).

Glioma specimens were obtained from surgical tumor resections. All studies were approved by the local ethics committee and written informed consent was obtained from all patients. The diagnosis was made according to the criteria of the WHO. Nonneoplastic brain tissue was harvested during surgeries for temporal lobe epilepsy. Samples were immediately snap frozen in isopentane, precooled over liquid nitrogen, and stored at −80°C until further processing.

Animals and tumor cell inoculation. For in vivo experiments, tumor cells were prepared and injected to mice as previously described (8, 11). Male SCID mice, ages 6 to 8 wk (Charles River Laboratories), were used for in vivo studies and were cared for in accordance with the standards of the Institutional Animal Care and Use Committee under a protocol approved by the Animal Care and Use Committee of St. Elizabeth Medical Center. Tumor volumes were recorded every 3 to 4 d. Tumor volume was calculated using the standard formula: length × width²/2 × 0.52.

Gene expression profiling. Genome-wide expression profiling was done using 51K Human Unigene III cDNA microarrays. The microarrays were designed, generated, and hybridized as previously described with minor modifications (13, 14). Linear amplification from 2 μg total RNA was done using the MessageAmp II cRNA Amplification Kit (Ambion). Amplified RNA (5 μg) was used for direct labeling (LabelStar Array Kit, Qiagen, #28904) by incorporation of Cy3-UTP or Cy5-UTP (Perkin-Elmer, NEL578;NEL579). The corresponding Cy3- and Cy5-labeled probes and competitor DNA (5 μg human Cot-DNA, Invitrogen; 5 μg poly(dA), Amersham) were combined, diluted in hybridization buffer to a final volume of 80 μL (50% formamide, 6× SSC, 0.5% SDS, 5× Denhardt’s solution), and denatured for 5 min at 95°C before hybridization. Prehybridization was done at 42°C for 20 min in 6× SSC, 0.5% SDS, 1% bovine serum albumin (BSA). Slides were rinsed in H2O and spotted probes were denatured by incubating the slide for 2 min in 90°C H2O. Hybridization probe was added and static hybridization was done at 42°C for 16 h. Excess probe was removed by washing in 2× SSC, 0.5% SDS at 42°C for 5 min; then in 0.2× SSC, 0.5% SDS at 42°C for 15 min; and finally in isopropanol for 30 s at room temperature.Slides were scanned with Agilent Microarray Scanner. Microarray data are available online at ArrayExpress under accession no. E-TABM-390.

Array data and pathway analysis. Generation of expression matrices, data annotation, filtering, and processing were done using our in-house TableButler software package. All microarray statistics including t test with permutation analysis (n = 1,000) and cluster analysis were done using the SUMO software package. The scanned chip images were processed using ChipSkipper software. Enrichment of genes among functional pathways and processes was analyzed as previously described (13, 14).

RNA extraction and PCR. RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s protocol. The integrity and concentration of RNA samples were determined by using RNA 6000 Nano LabChip kits and an Agilent 2100 Bioanalyzer. RNA (2 μg) was subjected to reverse transcription reaction. Reverse transcription reactions were carried out using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s protocol. Real-time PCR was done using TaqMan Gene Expression Assay (Applied Biosystems) kits. Assays were used for insulin-like growth factor binding protein-5 (IGFBP5) Hs00181213-M1, thrombospondin (THBS1) Hs00170236-M1, EGFR Hs00193306-m1, ESM-1 (ESM1) Hs00199831-m1, proline hydroxylase 1 (P4H1) Hs00165875, EphA5 Hs00300724, angiaptin (AMOT) Hs00611696-m1, transforming growth factor β2 (TGFβ2) Hs00234244-m1, tropomyosin-1x (TPM1) Hs00165966-m1, CD73 Hs00159686-m1, PDK (PDK3) Hs00178872, tissue inhibitor of metalloproteinase 3 (TIMP3) Hs01615949-m1, and IGF (IGF-R) Hs00181385-m1. PCR amplifications were done as multiplex, together with 18S probe as an internal control. For low copy number genes, single PCRs were done. Results were then normalized according to the internal control B2M VIC/MGB probe (Applied Biosystems).

Reverse transcription-PCR (RT-PCR) was used to evaluate the expression of EphA5 mRNA in human surgical specimens. Total RNA was isolated from tumor tissue using the RNeasy kit (Qiagen) and subjected to reverse transcription using poly(dt) primers. Dilutions of cDNA samples and numbers of PCR cycles have been evaluated to ensure nonsaturating PCR conditions. The RT-PCR experiments were done in double triplicates. The primer sequences were as follows: EphA5 forward, GTAGAGGAGGCTC-TATCGTCTE; EphA5 reverse, GAAATCTCTGTATACCGGC. The primers were annealed at 53°C.

Western blots and immunostaining. The following antibodies were used for Western blot: antibody for EphA5 (Santa Cruz, sc-1014); rabbit polyclonal antibody for the last 20 amino acids of angiotatin COOH terminus, provided by Dr. Holmgren (Karolinska Institute, Stockholm, Sweden; ref. 15); antibody for tissue inhibitor of metalloproteinase 3 (TIMP3; Chemicon, MAB3318); antibody for thrombospondin (A6.1; NeoMarkers); antibody for tropomyosin (Sigma, T2780); ESM-1 (c-20) antibody (Santa Cruz Biotechnology); antibodies for EGFR (sc-03) and e-EGFR (Tyr1173; Santa Cruz Biotechnology); and antibodies for IGF-IR (β) antibody (#3027) and phospho-IGF-IR (β) antibody (#3021B; Cell Signalling). For analysis of protein level in conditioned medium, cells were seeded in 175 flask (2 × 10⁶ per flask) in complete medium. On day 2, the cells were washed twice with serum-free medium and maintained in serum-free medium. After 18 h, the conditioned growth media were concentrated using Amicon Ultra-15 (Millipore). Protein concentrations were adjusted using Microcon centrifugal filter devices (Millipore). Protein concentration in the conditioned medium was quantified using Bradford assay and a total of 10 to 50 μg proteins for each sample were subjected to Western blotting.

For immunofluorescence staining, cells were fixed in 2% paraformaldehyde in PBS for 15 min at room temperature followed by three washes with PBS. For tropomyosin and EphA5 staining, cells were permeabilized with methanol in PBS for 20 min. Subsequently, cells were stained with the primary antibodies anti-tropomyosin, anti-EphA5, anti-angiotatin, and Fc-antigistatin diluted in PBS-1% BSA for 2 h at room temperature. Samples were then incubated with goat anti-mouse Alexxa 488 (Molecular Probes, #A11029), goat anti-mouse Alexa 555 (Molecular Probes, #A21424), goat anti-rabbit Alexa 555 (Molecular Probes, #A31629) or FITC-conjugated antihuman IgG antibodies (Fc specific; Sigma) diluted in PBS-10% goat serum for 1 h at room temperature. After washing in PBS, cells were washed in a 1:50 dilution of the nuclear dye To-Pro-III (Molecular Probes, #MP03602) for 10 min at room temperature in the dark. After washing in PBS and incubating in 300 nmol/L of the nuclear dye 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, #MP01306) for 5 min, cells were mounted in Vectashield mounting medium (Vector Laboratories, Inc.). Images were acquired using a fluorescence microscope (TE2000, Nikon) or a Zeiss Multispectral 510 META laser scanning confocal microscope.

Statistical methods. Data are presented as mean ± SE unless otherwise noted. Statistical significance was assessed using Student’s t test unless otherwise noted. P < 0.05 was considered statistically significant. All statistical tests were two tailed.

Results

Gene expression profiling of experimental models. In this work, we used human glioblastoma, osteosarcoma, liposarcoma,
Figure 1. Characterization of the experimental model. A, left, schematic presentation of the experimental model. Pairs of fast-growing or dormant tumor cell lines were generated from various tumor types. Whereas fast-growing tumor cells (red) generate tumors that expand rapidly, injection of dormant tumor cells (gray) resulted in occult lesions that were difficult to be detected by gross examination. Bar, 1 mm. The pictures are of tumors injected s.c. on the right flank of SCID mice. The top picture is of dormant glioblastoma, 111 d after the injection of the tumor cells. The bottom picture is of an angiogenic, fast-growing osteosarcoma, 48 d after the injection of the tumor cells. Right, schematic presentation of the in vivo tumor growth patterns of the human tumor models used. Red lines, growth of tumors generated from the fast-growing tumor cell lines; gray lines, tumors generated from the dormant tumor cell lines. These lines represent the earliest time point at which a tumor is detected after a s.c. tumor cells injection into the right flank of SCID mice. B, tumor sizes at experimental end points. There is a significant difference in the size of dormant tumors (gray columns) compared with fast-growing tumor cell lines (red columns). Dormant tumors at these time points were undetectable by gross examination (size values are zero). Each column represents one tumor. C, experimental strategy for the identification of the consensus gene expression signature of dormant tumors. Total RNA from each cell line was extracted and hybridized to genome-wide microarrays. Gene expression in every dormant tumor cell line (gray; D) was compared with that from the angiogenic fast-growing cell line (red; A) of the same tumor type. Each assay was done in duplicate and therefore is represented as two columns in the heat map. The heat map includes only genes that have the same pattern of expression in all tumor types analyzed. D, the top 10 functional processes differentially regulated between dormant versus fast-growing tumors. Data analysis of differentially regulated genes, in dormant versus fast-growing tumor cells, indicates the most significant enrichment for genes related to the regulation of angiogenesis process. Bars represent $-\log P$ values for the probability of enrichment for a gene ontology process arising by chance. Bars are colored based on the direction of gene regulation: red, up-regulated in dormant tumors; blue, up-regulated in fast-growing tumors.
and breast carcinoma tumor dormancy models. The phenotypic conversion of dormant tumors to fast-growing tumors after prolonged time of tumor dormancy was consistent and has been well characterized (8, 11, 16). Injection of the fast-growing tumor cell population from each tumor type resulted in generation of tumors that can be detected by gross examination 20 to 40 days following tumor cell inoculation. These rapidly growing tumors were well vascularized and "angiogenic" (Fig. 1A and B). In contrast, injection of dormant tumor cells consistently resulted in microscopic tumor that remained occult for a prolonged period of time (Fig. 1A and B). Once tumors emerge from dormancy, they grow in similar growth rates to the fast-growing tumors.

To detect differential expression of genes on a genome-wide scale, we generated and used the Human Unigene III c-DNA microarrays (13, 14). These chips consist of 51,000 elements and cover approximately >90% of the human genome. We compared gene expression patterns between dormant and fast-growing tumor cells from each tumor type (Fig. 1C). We searched for genes that showed the same pattern of expression in all dormant tumors versus fast-growing tumors. We found 430 transcripts that were down-regulated in dormant tumors compared with their fast-growing counterparts (Fig. 1C). Conversely, 381 transcripts were found to be up-regulated in dormant tumors compared with their fast-growing counterparts.

Importantly, an analysis of enriched gene ontology processes showed the angiogenesis pathway to be the most differentiated between the dormant and the fast-growing tumor phenotypes (Fig. 1D). This confirmed on the transcriptional level the important role of angiogenesis as a mechanistic basis for the phenotypic differences observed between dormant and fast-growing tumors.

**Validation using real-time PCR analysis.** From the genes that showed a similar pattern of expression regardless of tumor type and a statistically significant difference in those patterns between fast-growing and dormant cell lines, we chose several genes with known relevance for tumor angiogenesis or tumor progression (Table 1). Real-time PCR was done to confirm the array results and evaluate their expression.

The expression pattern of genes whose expression was up-regulated in at least three of the four dormant tumor cell lines is shown in Table 1. This analysis confirmed the up-regulation of thrombospondin, angioginotin, tropomyosin, transforming growth factor β2 (TGF-β2), and insulin-like growth factor binding protein 5 (IGFBP-5) in all dormant tumor cell lines. EphA5 expression was significantly increased in the dormant glioblastoma and, to a lesser extent, in the dormant liposarcoma. The most significant increase in expression was seen for the histone H2BK in the dormant glioblastoma, with the ratio of expression between the dormant and angiogenic fast-growing cell lines >2,000-fold (which exceeds the sensitivity level of real-time PCR). Proline 4-hydroxylase α polypeptide 1 (proline hydroxylase 1) expression increased in only three of four dormant tumor cell lines.

5'-Ecto-nucleotidase (CD73), endothelial cell–specific molecule 1 (ESM-1), PI3K catalytic β polypeptide (PI3-kinase p110 subunit beta), and TIMP-3 expression were consistently up-regulated in all angiogenic cell lines (Table 1). Several protein kinases were up-regulated in angiogenic fast-growing tumor cells. mRNA levels of EGFR, IGF-IR, and PI3K were moderately increased in angiogenic cell lines examined by real-time PCR. IGFR was not increased in the angiogenic liposarcoma cells.

**Comparison of protein levels in dormant and fast-growing tumor cells.** We analyzed the protein expression of the genes of interest in cells grown in tissue culture by Western blots (Fig. 2). Consistent with the RNA data, tropomyosin expression was elevated in dormant tumor cell lines with only minor differences between the liposarcoma clones (Fig. 2A).

At least two isoforms of angiogininotin (p130 and p80) have previously been identified (15, 17). Both isoforms are up-regulated in the dormant glioblastoma, osteosarcoma, and breast carcinoma.

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| Table 1. Real-time PCR of selected genes |
|-------------------------------|-----|-----|-----|-----|
|                               | Giloblastoma | Breast carcinoma | Osteosarcoma | Liposarcoma |
| (A) Genes up-regulated in dormant tumors |
| Thrombospondin (THBS1)       | 1.84 ± 0.01 | 4.86 ± 0.57 | 27.65 ± 2.19 | 2.54 ± 0.46 |
| Tropomyosin (TPM1)           | 3.74 ± 0.14 | 1.65 ± 0.07 | 3.13 ± 0.09 | 1.01 ± 0.05 |
| Angiomotin (AMOT)            | 13.15 ± 0.65 | 1.36 ± 0.02 | 17.58 ± 1.69 | 2.67 ± 0.34 |
| TGF-β2                       | 11.51 ± 1.93 | 1.98 ± 0.4 | 13.29 ± 3.49 | 1.53 ± 0.08 |
| Proline hydroxylase 1 (P4HA1)| 0.78 ± 0.02 | 1.15 ± 0.15 | 1.02 ± 0.02 | 2.36 ± 0.35 |
| EphA5                        | 299.56 ± 82.76 | 1.86 ± 0.21 | 1.11 ± 0.02 | 43.51 ± 11.49 |
| H2BK                         | 4.545 ± 548 | 1.58 ± 0.05 | 4.05 ± 0.86 | 1.04 ± 0.02 |
| IGFBP5                       | 8.96 ± 0.45 | 2.53 ± 0.05 | 14.19 ± 0.71 | 22.51 ± 1.42 |
| (B) Genes up-regulated in angiogenic fast-growing tumors |
| CD73                         | 2.91 ± 0.12 | 1.38 ± 0.06 | 3.67 ± 0.35 | 7.31 ± 0.34 |
| TIMP3                        | 1.31 ± 0.03 | 4.57 ± 0.11 | 1.31 ± 0.02 | 2.02 ± 0.04 |
| PI3K (PIK3CB)                | 1.25 ± 0.11 | 1.23 ± 0.05 | 1.42 ± 0.03 | 1.11 ± 0.16 |
| EGFR                         | 1.41 ± 0.18 | 1.55 ± 0.15 | 1.08 ± 0.16 | 1.11 ± 0.22 |
| ESM1                         | 32.05 ± 5.24 | 1.42 ± 0.08 | 3.35 ± 0.59 | 29.55 ± 1.02 |
| IGFR                         | 1.47 ± 0.11 | 1.35 ± 0.06 | 1.67 ± 0.05 | 0.58 ± 0.01 |

NOTE: (A) Gene expression in dormant tumor cells as compared with that in angiogenic cells. (B) Gene expression in angiogenic tumor cells as compared with that in dormant tumor cells.
Figure 2. Comparison of protein levels. Protein expression in cell lysates or conditioned medium was compared using Western blots. Protein levels in angiogenic fast-growing tumor cell lines (red columns and red gel lanes; A) and in dormant tumor cell lines (gray columns and gray gel lanes; D). Tropomyosin (A) and angiomotin (B) expression levels in cell lysates were quantified and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control for protein loading. Graphs present the ratio of tropomyosin or angiomotin protein levels in dormant tumor cells compared with that in angiogenic fast-growing tumor cells. TIMP-3 (C) and ESM-1 (D) expression levels in concentrated conditioned medium were determined and shown as compared with those in dormant tumor cells. Quantification of bands with low intensity was done using films that had longer exposure to the gel.

cell lines (Fig. 2B). Interestingly, an additional fragment of angiomotin with a molecular weight of 70 kDa has been observed in the dormant osteosarcoma and glioblastoma cells.

TIMP-3 protein expression was detectable both in cell lysates (data not shown) and in concentrated conditioned medium (Fig. 2C). Two forms of the protein were detected (p24 and p27). Whereas p24 is the predominant form in cell lysates (data not shown), p27 is the major secreted form.

ESM-1 is a secreted proteoglycan and was found only in conditioned medium (Fig. 2D). Although its RNA is up-regulated in all angiogenic cells examined, the posttranslationally modified form (50 kDa) was found only in conditioned medium of the breast carcinoma, liposarcoma, and osteosarcoma cells and was undetectable in the glioblastoma cells.

Among the genes up-regulated in angiogenic fast-growing tumor cell lines were several tyrosine kinases. Total EGFR protein levels were significantly elevated in angiogenic fast-growing glioblastoma and breast carcinoma cell lines (Fig. 3A). Binding of ligands to the EGFR activates an autophosphorylation process; therefore, activated receptors are found in their phosphorylated form. Interestingly, all cell lysates of angiogenic cell lines have higher levels of phosphorylated EGFR compared with dormant tumor cells (Fig. 3A).

IGFR total protein level was increased only in the angiogenic glioblastoma and osteosarcoma cell lines (Fig. 3B). However, high levels of phosphorylated IGFR are found in three of four angiogenic fast-growing tumor cell lines (Fig. 3B) including the angiogenic liposarcoma cells. Similarly to phosphorylated EGFR, phosphorylated IGFR can be found in cell lysates without the addition of ligands to the cells before analysis.

PI3K is well known to play a role in cancer. Although its RNA levels were shown to be up-regulated in all angiogenic
fast-growing tumor cell lines (Table 1), its protein could be detected only in the liposarcoma, glioblastoma, and osteosarcoma cell lines (Fig. 3C).

**Analysis of tropomyosin and EphA5 cellular localization.**

Next, we sought to determine whether there are differences in the cellular localization of the proteins analyzed. We stained the dormant and fast-growing tumor cell lines using tropomyosin-specific antibodies to determine if the tropomyosin protein localization in the cell is similar. Whereas most of the protein is found in the cytoplasm, some of it seems to be associated with the cell membrane (Fig. 4A, left). These results suggest that at least some of the cellular tropomyosin could be available for binding of extracellular proteins.

EphA5 belongs to the Eph subfamily of receptor protein tyrosine kinases. Similar to previously published data (18), EphA5 expression was found to be localized mostly in the cytoplasm rather than in the cell membrane (Fig. 4A, right). EphA5 was often aggregated in the vicinity of the tumor cell nuclei, but was also distributed in the cell cytoplasm with no apparent preference to the cell membrane.

**Angiostatin-mediated inhibition of migration of dormant tumor cells.** Angiostatin was previously described as an angiostatin binding protein. Indeed, we observed an exclusive binding of angiostatin to tumor cells that express angiostatin (Fig. 4B). Interestingly, although angiostatin and angiostatin can be found in the same cells, they do not colocalize (Fig. 4C).

Angiostatin was previously shown to inhibit the migration of angiostatin-expressing endothelial cells (19). We therefore sought to test whether angiostatin would affect the migration of tumor cells. Interestingly, angiostatin was shown to inhibit migration of tumor cells, but only of the dormant osteosarcoma and glioblastoma cells, and had no apparent effect on the migration of fast-growing angiogenic tumor cells (Fig. 4D).

**EphA5 levels in plasma and in tumor tissues correlate with tumor stage.** Although EphA5 belongs to a family of transmembrane receptor proteins, we found EphA5 in plasma (Fig. 5A) and
platelets (data not shown) of SCID mice. Interestingly, EphA5 levels significantly decreased in plasma of mice bearing angiogenic fast-growing glioblastoma. Importantly, a significant increase in EphA5 levels was evident in mice bearing microscopic dormant glioblastoma. EphA5 levels in mice carrying dormant liposarcoma were also increased compared with control mice (data not shown). However, this difference was not as pronounced as in the glioblastoma-bearing mice and was not statistically significant.

To further validate the correlation of EphA5 expression with tumor stage, we examined EphA5 expression in glioma tumors taken from human patients. Elevated EphA5 expression was detected by semiquantitative PCR in normal control brain tissues (Fig. 5B). EphA5 expression was decreased in low-grade glioma tumor tissues and was further reduced in high-grade glioma tumor tissues. Therefore, EphA5 expression is decreased with progression of tumor growth in glioma patients.

**Discussion**

In this study, the genetic participants of human tumor dormancy were identified by genome-wide transcriptional analysis of dormant tumors and their fast-growing counterparts. We found angiogenesis-related genes to be the most significantly affected functional gene category within the consensus dormancy signature. This does not exclude the involvement of additional pathways and mechanisms in cancer dormancy but rather strengthens the importance of angiogenesis blockage in maintaining tumors in a harmless state.

Among the genes that were up-regulated in all dormant tumors, thrombospondin is one of the best-characterized angiogenesis inhibitors. We further show that the PI3K RNA levels were 14% to 44% increased in fast-growing angiogenic glioblastoma, osteosarcoma, and breast carcinoma. We previously reported that the increase in thrombospondin expression in nonangiogenic cells is mediated via activation of the PI3K/c-Myc pathway (8). Taken together, it seems that the PI3K-mediated decrease in thrombospondin level is a common mechanism used by angiogenic fast-growing tumor cells.

We found elevated angiomotin protein levels in dormant tumor cells. Angiomotin was found to mediate the antiangiogenic activity of the endogenous angiogenesis inhibitor angiostatin (17, 20), which was shown to suppress tumor growth and maintain dormancy of tumor metastases (21, 22). Here we show that angiostatin preferentially binds to dormant tumor cells that express high levels of angiomotin. However, we found no evidence for angiostatin-angiomotin interaction or colocalization within the...
dormant tumor cells. Therefore, it is most likely that additional proteins or mediators are involved in this process. Although angiostatin was previously shown to selectively affect endothelial cells, we show here that it also inhibits migration of dormant tumor cells.

The Ephrin family of receptor tyrosine kinases and their ligands are known to be involved in embryonic and adult neurogenesis and angiogenesis (13, 23). We found elevated EphA5 RNA levels in all dormant tumor cell lines tested. Moreover, in glioma patients, the expression of EphA5 correlated with pathologic tumor grade. Although assumed to be a membrane receptor protein, we observed distinct patterns of circulating EphA5 plasma levels of control and tumor-bearing mice. Importantly, EphA5 plasma levels were significantly increased in mice bearing occult, dormant tumors. The observation that EphA5 levels decrease as the tumor stage advances in glioma tumors (Fig. 5B) suggests a novel role for EphA5 as a tumor suppressor protein.

We also found higher expression levels of tropomyosin in dormant tumors. Tropomyosin was previously shown to bind and mediate the antiangiogenic properties of another endogenous angiogenesis inhibitor, endostatin (24–26). In agreement, we show that tropomyosin is also localized on the cell membrane surface and, therefore, could possibly bind to endostatin in the tumor microenvironment.

Prolyl hydroxylation is a rate-limiting step in collagen biosynthesis. The up-regulation of the α(I) isoform (the major form) was confirmed by real-time PCR. Collagen prolyl-4-hydroxylase α(II) was previously shown to mediate the extracellular release of antiangiogenic fragments of collagen IV and collagen XVIII and to significantly attenuate in vivo tumor growth in mice (27).

Histone H2BK, a core component of the nucleosome, was highly up-regulated in all dormant tumor cells analyzed in this study. Although histone acetylation is well known to affect the angiogenesis process, the role of histone H2BK in tumor progression is still unclear.

The insulin-like growth factor axis has emerged as an important pathway in cancer (28, 29). The availability of IGF to its receptor is controlled by the interactions with IGFBPs. The fact that two opposing players in the insulin-like growth factor pathway had inverse patterns of expression in dormant versus fast-growing tumors suggests that activation of the IGF pathway might play a critical role in the conversion of dormant tumors to the fast-growing angiogenic phenotype.

Another consistently up-regulated gene in all fast-growing tumors was CD73/5′-ecto-nucleotidase. CD73 is a cell membrane–anchored surface enzyme that catalyzes the extracellular conversion of 5′-AMP to purine nucleoside adenosine and, therefore, controls many physiologic events (30). Adenosine is known to accumulate in solid tumors and to stimulate tumor growth and angiogenesis (31).

ESM-1, or endocan, originally described as a proteoglycan secreted by cultured endothelial cells (30), is highly expressed in tumor endothelium (32) but can also be found in tumor cells. Here we show that ESM-1 is significantly elevated in all angiogenic fast-growing tumor cells analyzed. Interestingly, ESM was shown to promote tumor formation and its levels were increased in cancer patients (33).

In summary, the genes identified in this study orchestrate the conversion of dormant tumors into fast-growing angiogenic tumors. Whether these genes and proteins directly contribute
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Received 7/7/2008; revised 10/10/2008; accepted 11/6/2008.
Grant support: National Aeronautics and Space Administration Specialized Center of Research grant NNJ04HH12G, Deutsche Krebshilfe grant 106997, Deutsche Forschungsgemeinschaft National Priority Research Program Tumor-Vessel Interface grant SPP1190, and Tumorzentrum Heidelberg-Mannheim.

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This article is dedicated to the memory of Dr. Judah Folkman who passed away while preparing the manuscript and to his inspirational teaching, remarkable intuitions, and passionate devotion to the field of tumor angiogenesis.

We thank Kashi Javaherian, Tong-Young Lee, Hui-Ya Gilbert, Afshin Beheshti, Giannoula Klement, and Janusz Waremowicz for help with technical issues and supplies; Lars Holmgren for providing the antibody for angiominot and helpful comments; Thuy Trinh for help with culture and microarray work; Kristin Johnson for help with graphics; and Phillip Hahnfeldt for critical reading of the manuscript.

References
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