Malignant Transformation of Human Cells by Constitutive Expression of Platelet-derived Growth Factor-BB*

Baskaran Govindarajan‡‡, Asha Shah‡, Cynthia Cohen‡, Rebecca S. Arnold‡, Jeffrey Schechner‡, Jun Chung‡, Arthur M. Mercurio‡, Rhoda Alani**, Byungwoo Ryu**, Chun-Yang Fan‡‡, Jose M. Cuezva‡‡, Marta Martinez‡‡, and Jack L. Arbiser††

From the ‡Departments of Dermatology, Pathology, and Laboratory Medicine, Emory University School of Medicine and Veterans Affairs Hospital, Atlanta, Georgia 30322, the ‡Department of Dermatology, Yale University, New Haven, Connecticut 06511, **The Sidney Kimmel Comprehensive Cancer Center and Department of Dermatology, The Johns Hopkins University, Baltimore, Maryland 21224, ††Beth Israel Deaconess Hospital, Division of Signal Transduction, Harvard Medical School, Boston, Massachusetts 02138, the ‡‡University of Arkansas for Medical Sciences and Veterans Affairs Medical Center, Little Rock, Arkansas 72205, and the §§Departamento de Biología Molecular and the Departamento de Matemáticas, Centro de Biología Molecular “Severo Ochoa,” Universidad Autónoma de Madrid, E-28049 Madrid, Spain

Platelet-derived growth factors (PDGFs) comprise a family of growth factors strongly implicated in human oncogenesis. A number of human tumors overexpress PDGF family members or have translocations activating PDGF receptors. Whereas the epidemiologic evidence implicating PDGF in human tumors is strong, malignant transformation of human cells by overexpression of PDGF has not been demonstrated. We have previously developed a human cell line by the sequential introduction of large T cells and telomerase, and we have demonstrated that these cells express functionally active PDGF receptor (PDGFR) β. In order to determine whether growth factor-mediated transformation of human cells could occur, these cells were transduced with a retrovirus encoding PDGF-BB. Constitutive expression of PDGF-BB led to malignant transformation in nude mice. This is the first demonstration of constitutive signaling causing malignant transformation of human cells. Some of the changes that occur because of constitutive growth factor expression can be reversed by the clinically approved tyrosine kinase inhibitor Glivec, whereas other changes are not reversible by tyrosine kinase inhibitors. Our model allows the assessment of epigenetic changes that occur during human carcinogenesis. In addition, these studies provide insight into the clinical failure of tyrosine kinase inhibitors as monotherapy for advanced malignancy.

The PDGF family of peptides includes PDGF A, B, and C, and these ligands are capable of heterodimerization (1, 2). The receptors for PDGF include PDGFRα and PDGFRβ, and stimulation of these receptors results in a number of signaling events, including induction of phosphoinositide-3-kinase, mitogen-activated protein kinase, and reactive oxygen-NFκB signaling. The first demonstration of PDGF in oncogenesis was the discovery that the transforming gene of the v-sis retrovirus is PDGF (1, 3, 4). More recently, PDGFR rearrangements have been demonstrated in a number of translocations, including dermatofibrosarcoma protuberans, in which a collagen type 1 transcript is fused to PDGFRβ, and myeloproliferative disorders, including the PDGFRα tel translocation (5–7). Despite the discovery of constitutive PDGF-mediated tumorigenesis in human tumors, the steps involved in multistep tumorigenesis because of PDGF signaling are not well understood.

We previously derived a cell line (SV7tert) from a human angiomyolipoma through the sequential introduction of SV40 large T cells and telomerase (8), and we subsequently demonstrated that PDGFRβ is present and active in these cells, as well as in angiomyolipomas, the benign tumors from which SV7tert cells were derived (9). Despite the introduction of two oncogenes into these cells, they fail to form tumors in nude mice. One potential reason for this failure is the presence of high levels of PDGF in the kidney but not the skin. Knockouts of PDGF or its receptors lead to severe defects in renal development, and these defects are the major cause of lethality in mice carrying these deficiencies (10, 11). In order to explore whether PDGF could cause these cells to become tumorigenic, we introduced constitutive expression of PDGF-BB using retroviral gene transfer into SV7tert cells.

Constitutive expression of PDGF-BB led to tumorigenesis in nude mice, with a prolonged latency of several months. This implied that epigenetic factors may play a role in PDGF-mediated tumorigenesis in human cells. Among the changes we observed in PDGF-transduced cells are high levels of reactive oxygen. We have shown previously (12) that carcinogenesis due to reactive oxygen results in silencing of the tumor suppressor p16INK4a. Consistent with this, we observed decreased expression of p16INK4a in tumor-derived tissue compared with the original PDGF-overexpressing cell line. The p16INK4a silencing gene Id-1 was highly expressed in tumor-derived tissue. Consistent with this hypothesis, tumorigenesis in vivo was accelerated by introduction of Id-1 into PDGF-expressing SV7tert cells. We observed that in vivo growth selects for cells with a
low bioenergetic index (BEC) (Warburg effect). Inhibition of PDGFRβ signaling with the clinically approved inhibitor Glivec resulted in inhibition of some but not all of the changes observed because of constitutive expression of PDGF, implying that PDGF-induced carcinogenesis of human cells results in both reversible and irreversible changes. These irreversible changes may account in part for the failure of tyrosine kinase-based therapies in advanced malignancies.

MATERIALS AND METHODS

Overexpression of PDGF-BB in SV7tert Cells

SV7tert cells were infected with retroviruses encoding PDGF-BB or GFP as a vector control according to the method of Scheckner and co-workers (13). Unselected pooled cells were used for further in vitro and in vivo analyses. PDGF overexpression was confirmed by enzyme-linked immunosorbent assay.

Regulation of VEGF mRNA and Protein

For the analysis of protein expression of VEGF, cells were extracted in RIPA buffer (20 mM Tris buffer, pH 7.4, containing 0.14 NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 2 mM PMSF, 5 μg/ml aprotinin, pepstatin, and leupeptin), and immunoblotting was performed as described previously (14). A rabbit anti-VEGF (A-20) was obtained from Santa Cruz Biotechnology; a rabbit anti-actin antibody was from Sigma. Immunohistochemistry was performed as described by Arbiser et al. (16).

Apoptosis Assay

Adherent and nonadherent cells were harvested and assayed for apoptosis using annexin V-fluorescein isothiocyanate and propidium iodide (Fig. 4) (Biosource, Sunnyvale, CA) as described previously (14).

In Vivo Tumorigenesis

SV7tert, SV7tert PDGF, SV7tert PDGF-pLXSN, and SV7tert PDGF-Id-1 (1 × 106) cells were injected into the flank of 6-week-old nude male mice (Charles River Breeding Laboratories). Three weeks after tumors became visible, they were excised and fixed in formalin for hematoxylin and eosin staining (Fig. 6). Tumor volume was measured using the formula (width2 × length) × 0.52, where width represents the shortest dimension (Fig. 5). Transduction of SV7tert cells with Id-1 in the absence of PDGF did not lead to tumorigenesis over a period of 6 months (data not shown).

p16 Expression Analysis in Tumor Tissue

RT-PCR for p16ink4a—Total RNA was isolated using TrizReagent (Sigma). RT-PCR was done with Promega RT-PCR kit (Fig. 7). Primers were designed as follows: actin (728 bp), forward 5′-AAG ATG ACC CAG ATC ATC CTT GCT 3′ and reverse 5′-CTG CTT GCT GAT CCA CAT CTG CTT G-3′; p16ink4a (196 bp), forward 5′-CGA TTG AAA GAA CCA GAG CAA CAG AC-3′ and reverse 5′-ATG GAC ATT TAC GGT AGT GG-3′. Reactions were performed in an Eppendorf master cycler. One PCR cycle at 45 °C for 45 min and 94 °C for 2 min followed by 40 PCR cycles under standard conditions with an annealing temperature of 60 °C were performed. β-Actin mRNA was used as a reference message to normalize the content of total RNA. p16ink4a expression was calculated as the relative expression ratio to that of β-actin. All reactions were carried out in triplicate. Quantification was determined by triplicate repeats.

In Vitro Proliferation Assays—10,000 cells were plated in 24-well plates. The next day, the medium was replaced with fresh medium containing the inhibitors or vehicle controls. Cells were incubated at 37 °C for 24 h, and cell number was determined in triplicate using a Coulter Counter (Hialeah, FL). Cells were treated with either vehicle (20 μl of Me2SO) or increasing concentrations (5, 10, 15, and 20 μM concentration) of Glivec from a 10 mM stock in Me2SO.

Methylation Analysis of p16ink4a

Bisulfite Modification of DNA for Methylation-specific PCR—DNA samples, negative (human placental DNA; Sigma) and positive (CpGenome™ universal methylated human DNA; InterGen Co., New York) controls, were subjected to bisulfite modification prior to methylation-specific PCR using CpGenome™ DNA modification kit (InterGen Co.).

PCR Amplification and Primers—Amplification of the promoter region of the p16 gene was carried out in a Touchgene Gradient Thermal Cycler (Techne Inc., Princeton, NJ) in a 50-μl PCR mixture containing 2 μl of bisulfite-treated genomic DNA, dNTPs (each at 200 μM), primers (50 pmol each per reaction), 2.5 mM MgCl2, and 1.25 units of Hotstar Taq (Qiagen, Valencia, CA) in 1× PCR buffer. All reactions were supplied with the Qiagen Hotstar Taq kit (Qiagen, Valencia, CA). The only exception was the dNTP mix (Roche Applied Science).

Primers for p16 gene were designed as follow: 5′-TTATTAGAGGGTGGGTTGATTTG-3′ (sense) and 5′-CAACCCCAACACCATCAT-3′ (antisense) for the unmethylated reactions; 5′-TTATTAGAGGGTGGGCGGCTGCTG-3′ (sense) and 5′-GACCCGGGCGCCCCGGCCGC-3′ (antisense) for the methylated reactions as described previously (38). Both primers were purchased from Operon Technologies (Alameda, CA). The PCR conditions were as follows: initial denaturation and hot start at 95 °C for 15 min, then 40 cycles consisting of 30 s at 95 °C, 30 s at 60 °C (unmethylated reactions) or 65 °C (methylated reactions), and 1 min at 72 °C followed by a final 5-min extension at 72 °C. Positive and negative control DNA samples and controls without DNA were used for each set of PCRs.

Introduction of Id-1 Accelerates Tumorigenesis in Vivo

SV7tert PDGF cells were infected with retroviruses encoding pLXSN or a constitutively active Id-1 (15). The unselected cells were pooled and were used for further in vitro and in vivo experiments. Overexpression of Id-1 was confirmed by Western analysis (Fig. 9).

For Id-1 immunoblot experiments, 30–40-μg aliquots of cell and tissue lysates prepared as above were separated by 15% SDS-PAGE and electroblotted to polyvinylidene fluoride membranes (Immobilon P, Millipore). The membrane was blocked and then incubated with primary antibody (Id-1 polyclonal antibody from Santa Cruz Biotechnology, SC-488) in TNF buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 50 mM NaCl, 0.1% Tween 20) overnight at 4 °C. Antibody-antigen complexes were detected and visualized after incubation for 1 h with appropriate horseradish peroxidase-labeled secondary antibodies (Am-
Measurement of BEC

The human angiomylipoma SV7tert cells (parental, PDGF, tumors 1 and 2) were grown in Dulbecco's modified Eagle's medium supplemented with a mixture containing 400 μM of each of the following amino acids: Ala, Asn, Asp, Glu, and Pro, and 1 mM Gln (Merck), plus 10% fetal calf serum (Biological Industries), 0.6% penicillin, and 1% streptomycin (Sigma) in 7% CO2 atmosphere (Fig. 10). Cells were recovered from the plates by trypsin treatment and washed twice with phosphate-buffered saline. The recovered cells were lysed in a buffer containing 5 mM Tris-HCl, pH 8.0, 0.5% Triton X-100, 20 mM EDTA, 1 μg/ml pepstatin, and 1 μg/ml leupeptin at 4 °C for 10 min. After protein extraction, the samples were centrifuged (15,000 × g) at 4 °C for 5 min. The protein concentration in the supernatants was determined with the Bradford reagent (Bio-Rad Protein Assay) using bovine serum albumin as standard. Aliquots of the supernatants were stored at −80 °C until used.

Cellular proteins (10 μg of protein) were fractionated on 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore). Western blotting of the membranes was essentially carried out as described previously using the appropriate dilution of various antisera. The antibodies used in this study are as follows: rabbit anti-β-F1-ATPase at a dilution of 1:20,000 (22); mouse monoclonal anti-hsp60 (SPA 807, StressGen, Victoria, Canada) at a dilution of 1:2,000; mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase at a dilution of 1:2,000 (Millipore). Western blotting of the membranes was essentially carried out as described previously using the appropriate dilution of various antisera. The antibodies used in this study are as follows: rabbit anti-β-F1-ATPase at a dilution of 1:20,000 (22); mouse monoclonal anti-hsp60 (SPA 807, StressGen, Victoria, Canada) at a dilution of 1:2,000; mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase at a dilution of 1:20,000; goat polyclonal anti-α-tubulin (Sigma) at a dilution of 1:2,000; polyclonal anti-muscle pyruvate kinase at a dilution of 1:2,000 and mouse monoclonal anti-glycerol dephosphorylase (Sigma) in 7% CO2 atmosphere (Fig. 10). Cells were recovered from the plates by trypsin treatment and washed twice with phosphate-buffered saline. The recovered cells were lysed in a buffer containing 5 mM Tris-HCl, pH 8.0, 0.5% Triton X-100, 20 mM EDTA, 1 μg/ml pepstatin, and 1 μg/ml leupeptin at 4 °C for 10 min. After protein extraction, the samples were centrifuged (15,000 × g) at 4 °C for 5 min. The protein concentration in the supernatants was determined with the Bradford reagent (Bio-Rad Protein Assay) using bovine serum albumin as standard. Aliquots of the supernatants were stored at −80 °C until used.

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PDGF Secretion Assay

PDGF secreted into the growth media was measured using Quantikine human PDGF-AB immunoassay kit from R & D Systems (catalog number DHD00B), and the assay was conducted according to the manufacturer's procedure. Immunohistochemical analysis of tumors for PDGF-BB and VEGF was carried out as described previously by Arbiser (16). Adaptive Oxygen Measurements

Hydrogen peroxide and superoxide levels were quantitated by the use of dichlorofluorescein and dihydroethidine fluorescence, respectively (Fig. 11), according to the method of Arbiser (17).
cells according to the method of Shaw et al. (18). Constitutive expression of PDGF-BB led to a significant induction of luciferase activity, indicating that a major mode of VEGF regulation by PDGF-BB in this system is at the level of transcription.

Because VEGF is highly regulated at the level of transcription, we examined the effects of signal transduction inhibitors of VEGF mRNA expression. The induction of VEGF mRNA by PDGF-BB was blocked by the phosphoinositol 3-kinase inhib-
Most surprisingly, treatment with the antioxidant N-acetylcysteine led to a slight induction of VEGF mRNA (Fig. 3). We then examined the effect of SV7tert parental cells and PDGF-expressing SV7tert to hypoxia. Exposure of parental SV7tert cells to hypoxia led to a robust induction of VEGF mRNA. SV7tert cells expressing PDGF showed an increased baseline expression of VEGF mRNA but a minimal increase because of hypoxia (Fig. 4). We also examined the effect of hypoxia on apoptosis in SV7tert and PDGF-expressing cells. Hypoxia decreased the rate of apoptosis in both cells, but the decrease in apoptosis was more marked in PDGF-expressing cells (Fig. 4).

**PDGF-BB Overexpression Causes Tumor Growth in Vivo**—
Equal numbers of SV7tert PDGF and vector control cells were implanted into nude mice. PDGF-expressing cells formed slowly growing but steadily progressive tumors. No tumors were observed in the vector control cells (Fig. 5). Histologically, tumors demonstrated pseudocapapsulation, with malignant cells infiltrating muscle and adipose tissue (Fig. 6). Mice were observed up to a period of 6 months, at which time mice were sacrificed. Abundant expression of PDGF-BB and VEGF was observed in vivo (Fig. 7).

**In Vivo Growth Selects for High Levels of PDGF Secretion**—
Results from the PDGF secretion assay on the different SV7tert cell lines prior to and after implantation show an increase in the levels of PDGF secreted in the media from tumor-derived cell lines. PDGF levels were calculated per million cells. PDGF radioimmunoassay shows the following levels of PDGF-BB secretion per million cells: assay shows SV7tert at 22827.4, and SV7tert PDGF at 8697.4 pg of PDGF/million cells. Thus, in tumor 1, the cells secreted over 15-fold more PDGF than cells prior to implantation, and in tumor 2, the tumor-derived cells secreted 5-fold more PDGF than cells prior to implantation. Thus, in vivo growth selects for high levels of PDGF secretion, providing compelling evidence for a role of PDGF-BB in in vivo tumorigenesis.

**p16<sup>ink4a</sup> Expression Is Decreased during in Vivo Growth**—
We have shown previously that loss of p16<sup>ink4a</sup> occurs in reactive oxygen-induced tumors and in spontaneous tumors in mice heterozygous for tuberin (12, 19). Thus, we hypothesized that loss of p16<sup>ink4a</sup> could occur during the long latency period of tumor formation in human cells. We examined expression of p16<sup>ink4a</sup> in parental, PDGF-expressing cells prior to implantation and in tumor tissue derived after in vivo growth. p16<sup>ink4a</sup> expression was markedly diminished in tumor tissue compared with cells prior to in vivo implantation (Fig. 8).

Hypermethylation and transcriptional silencing have been established as common mechanisms for the decreased expression of p16<sup>ink4a</sup> in human tumors. Analysis of the p16<sup>ink4a</sup> promoter failed to reveal evidence for p16<sup>ink4a</sup> hypermethylation (data not shown). We then examined the expression of proteins known to silence p16<sup>ink4a</sup> expression, namely BMI-1 and Id-1. BMI-1 expression is increased in the SV7tert cells expressing PDGF prior to implantation but is decreased in tumor-derived cells, making it unlikely that BMI-1 is the sole mediator of p16<sup>ink4a</sup> silencing (Fig. 9). In contrast, the helix-loop-helix protein Id-1 is expressed in tumor-derived tissue (data not shown). In order to see whether forced expression of Id-1 had a functional effect on tumorigenesis, SV7tert PDGF and parental cells were infected with a retroviral vector encoding Id-1 or a control retroviral vector. Tumorigenesis was enhanced by the co-expression of Id-1 and PDGF-BB (Fig. 10). Expression of Id-1 alone was insufficient to cause malignant transformation of SV7tert cells in vivo (data not shown).

**In Vivo Growth Selects for Cells with Glycolytic Metabolism (Warburg Effect)**—Highly aggressive tumor cells have long been known to suppress respiration in favor of glycolysis, but the reasons for this switch remain unclear (20, 21). Cuezva et al. (22) have demonstrated that tumors that have a high level of glycolysis have a poor prognosis and have quantitated BEC as a ratio of mitochondrial to glycolytic enzymes, as a measure of glycolytic metabolism. Cells with a high BEC have a high level of respiration, and cells with a low BEC are associated with glycolytic metabolism and poor prognosis. SV7tert PDGF cells prior to implantation show a high BEC, whereas tumor-
derived cells show a decreased BEC, consistent with a shift to glycolytic metabolism (Fig. 11). Thus, in vivo growth selects for cells with a glycolytic metabolism and suggests that glycolysis or suppression of respiration may enhance tumor growth in vivo. In order to see whether the changes in glycolysis were dependent on PDGF-BB, we repeated the glycolytic flux observations in the presence and absence of Gleevec. Most surprisingly, Gleevec treatment caused increased glycolysis in all the PDGF-BB-secreting cell lines (Fig. 12). In addition, Gleevec treatment inhibited proliferation in all cell lines in a dose-dependent fashion (Fig. 13), indicating that these activities are dependent in part on sustained production of PDGF-BB.

PDGF-BB is known to activate reactive oxygen species acutely, but the chronic effects of PDGF stimulation are not well characterized. We examined the expression of hydrogen peroxide and superoxide in parental PDGF-expressing and tumor-derived cells. Of interest, cells prior to implantation revealed high level expression of both hydrogen peroxide and superoxide. However, tumor-derived cells have decreased levels of superoxide, yet have persistently elevated levels of hydrogen peroxide (Fig. 14). Treatment of all cell lines with the clinically approved PDGFRβ tyrosine kinase inhibitor Gleevec did not result in any changes in reactive oxygen species (data not shown).

**DISCUSSION**

Rapid transformation of human cells has been accomplished by introduction of telomerase, large T antigen, and Ras (23). These studies elegantly demonstrate the steps required for full transformation of human cells and allow the assessment of epigenetic events that occur over long term human carcinogenesis. We have previously established a cell line (SV7tert) from an angiomylipoma, and we found that it expresses functional PDGFRβ (8, 9). In order to determine whether autocrine PDGF-PDGFRβ signaling could result in malignant transformation, we retrovirally transduced PDGF-BB into SV7tert cells. Overexpression of PDGF-BB led to up-regulation of vascular endothelial growth factor, and reactive oxygen species, as well as resistance to apoptosis under hypoxic conditions. Implantation of SV7tert cells expressing PDGF-BB led to the development of tumors in nude mice, whereas tumors were not observed in vector control cells. Tumors took several months to appear, suggesting further epigenetic events.

Investigations from our laboratory and others suggest a linkage between inactivation of specific transcription factors and activation of specific signaling pathways. Our initial studies demonstrated that in a model p53-deficient system, angiosarcoma and phosphatidylinositol 3-kinase activation are required for tumorigenesis, but MAP kinase is not required (24, 25). Further studies have revealed a linkage between reactive oxygen and MAP kinase in p16-deficient tumors, with phosphatidylinositol 3-kinase playing an additional role in angiogenesis and in the prevention of apoptosis (26–29). In tumors derived from mice heterozygous for tuberin, we have noted an obligatory role of MAP kinase for tumorigenesis, as well as loss of p16 in these tumors (19). We introduced PDGF-BB into human angiomylipoma cells, which contain a functional PDGFRβ, in order to determine whether these cells could undergo autocrine transformation. Consistent with our predictions, PDGF overexpression was transforming in human cells. This overexpress-
sion was accompanied by increased production of reactive oxygen species. We thus predicted, based upon prior studies, that p16<sup>ink4a</sup> expression would be decreased in cells that were derived from tumors compared with cells prior to implantation. It is thus likely that the process of in vivo growth selects for decreased expression of p16<sup>ink4a</sup>. In order to determine the mechanism of decreased p16 expression, we performed methylation analysis of genomic DNA from cells prior to and after tumor formation. Hypermethylation did not occur. A second mechanism of p16 silencing is through promoter-binding proteins. Among these proteins are the transcription factor Id-1 and the polycomb protein BMI-1 (30, 31). BMI-1 is up-regulated as an early event in PDGF-induced tumorigenesis, and expression is decreased in tumor-derived cells, whereas Id-1 is highly expressed in PDGF-induced tumors. Consistent with this observation, Id-1 overexpression accelerated PDGF-induced tumorigenesis in vivo.

Targeted therapies, including Glivec, Iressa, and Herceptin, are finding increased use in cancer therapy. Despite this, many tumors are resistant to these therapies, despite the fact that relevant tyrosine kinases are activated in the tumor cells. Our findings provide a potential explanation for the failure of targeted therapies. We demonstrate that constitutive expression of a growth factor can cause malignant transformation of human cells. We also demonstrate that the tyrosine kinase inhibitor, Glivec, exhibits biological activity against cells prior to and after selection for in vivo growth. However, not all of the changes that occur after transformation can be reversed by Glivec, implying that metabolic changes occur that gain independence from growth factor stimulation. Most surprisingly, glycolysis was potentiated by Glivec in PDGF-secreting tumors. Tumor-derived cells are selected for glycolytic growth, so the potentiation of glycolysis by Glivec may explain in part the lack of efficacy of Glivec in highly advanced tumors. Glivec may be more efficacious against earlier tumors that have a greater dependence on respiratory metabolism. Indeed, one form of Glivec resistance in chronic myelogenous leukemia is associated with altered mitochondrial function (32).

Aggressive tumor cells have been demonstrated to use glycolysis as a predominant mode of metabolism. This observation, known as the Warburg effect, is not fully understood nor is the survival advantage conferred by glycolysis (33). However, human tumors with a low BEC, a marker of glycolytic metabolism, have a poor prognosis (22). In order to determine whether in vivo growth selected for this phenotype, we compared the bioenergetic index of cells prior to and after implantation. The BEC of cells from two different tumors was greatly diminished from parental SV7tert PDGF cells. Thus, it is likely that the shift to a glycolytic metabolism supplies a growth advantage in vivo. The precise survival advantage of the Warburg effect is not fully understood, but glycolytic cells have been shown to have increased resistance to hydrogen peroxide-induced apoptosis (34, 35). This may be of relevance to our tumors, which have persistently elevated levels of hydrogen peroxide.

Overexpression of a growth factor or its receptor is a common event in human tumorigenesis (36, 37). However, the precise role of growth factor overexpression in human tumorigenesis is not fully understood. Neoplasms known to have aberrant growth factor overexpression include multiple myeloma and melanoma, neoplasms in which p16 inactivation is a common event. Whereas blockade of growth factor expression can be accomplished by gene silencing techniques such as small interfering RNA, these techniques do not recapitulate the epigenetic changes that are required for in vivo growth due to continuous growth factor activation. We demonstrate for the first time autocrine transformation of human cells in vitro, and we demonstrate the epigenetic changes that occur in in vivo growth, namely silencing of p16 and increased glycolysis. Chemoprevention and treatment of these malignancies may be accomplished by therapies that decrease reactive oxygen and/or prevent transition to glycolytic metabolism.

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