Yin Yang-1 inhibits tumor cell growth and inhibits $p21^{WAF1/Cip1}$ complex formation with Cdk4 and cyclin D1

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Abstract. The GLI-Krüppel zinc finger factor yin yang-1 (YY1) is a complex protein that regulates a variety of processes including transcription, proliferation, development and differentiation. YY1 inhibits cell growth in a cell type-specific manner. The role played by YY1 in its control of tumor cell growth is unclear and controversial. We show here that YY1 can suppress the growth of different tumor cell types *in vitro*, including human breast carcinoma cells and glioblastoma cells. YY1 also blocked the growth of 13762 MAT mammary adenocarcinoma isografts in rats. YY1 inhibited 13762 MAT tumor growth by approximately 80% compared with the GFP alone group 21 days after injection. YY1 inhibited proliferating cell nuclear antigen (PCNA) expression and $pRb^{Ser249/Thr252}$ phosphorylation without influencing tumor microvascular density. Moreover, YY1 inhibited $p21^{WAF1/Cip1}$ complex formation with cdk4 and cyclin D1. These findings demonstrate that YY1 can negatively regulate the growth of multiple malignant cell types.

Introduction

Yin yang-1 (YY1) is a ubiquitous and multifunctional transcription factor (also known as delta, NF-E1, UCRBP, and CF1) belonging to the C_2H_2 subclass of GLI-Krüppel zinc finger proteins. YY1 was cloned and characterized initially by two independent groups (1,2). The YY1 gene resides on human chromosome 14 at segment q32.2 and consists of five highly-conserved exons which undergo alternative splicing and encode four C_2H_2 motifs (3). YY1 protein contains both activation and repression domains at the amino-terminus and carboxyl-terminus, respectively. YY1 plays a role in transcriptional initiation, activation and repression (4). Physiologically, YY1 is required for a variety of processes including embryogenesis, growth and differentiation (4). However, the role of YY1 in cancer growth is poorly defined.

We previously demonstrated that YY1 is induced by mechanical injury of rat arteries and exposure of vascular smooth muscle cells to growth factors such as fibroblast growth factor-2 (5). YY1 overexpression inhibited vascular smooth muscle cell growth in culture (5) and intimal hyperplasia in rat, rabbit and human blood vessels (6). Interestingly, YY1 does not appear to influence vascular endothelial cell growth (5,6). The role played by YY1 in its control of tumor cell growth is unclear and controversial.

Here we demonstrate that YY1 can suppress the growth of different tumor cell types *in vitro,* including human breast carcinoma cells and human glioblastoma cells. Moreover, YY1 blocked solid tumor growth in rats, inhibited proliferating cell nuclear antigen (PCNA) expression and $pRb^{Ser249/Thr252}$ phosphorylation without influencing tumor microvascular density. These findings show that YY1 can negatively regulate the growth of multiple malignant cell types.

Materials and methods

Cell proliferation assays. Human MCF7 breast carcinoma cells, human U178 glioblastoma cells and rat 13762 MAT mammary adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC) and grown in RPMI-1640, pH 7.4, containing 10% FBS with antibiotics (7). Cells in 96-well plates (3000 cells seeded), were rendered growth quiescent by incubation in serum-free medium for 24 h. The cells were transfected with the indicated amounts of plasmid or transduced with the indicated amounts of adenoviral constructs, Ad-LacZ or Ad-YY1 (6) in 10% FBS/DMEM, pH 7.4. After 2 or 3 days (as indicated), the cells were trypsinized, resuspended in Isoton II and quantified using a Coulter counter.

Western blot analysis. Samples were resolved by electrophoresis using denaturing SDS-polyacrylamide gels for 2 h at 100 V. Proteins were transferred to Immobilon P nylon membranes (Millipore) prior to incubation with non-fat

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skim milk to block non-specific binding sites. Membranes were incubated with the indicated antibodies (Santa Cruz Biotechnology). Detection was achieved with HRP-linked secondary antibodies at a dilution of 1:1000 and chemiluminescence (Perkin-Elmer).

Construction of retroviral YY1, transduction of 13762 MAT mammary adenocarcinoma cells and primary tumor growth in rats. YY1 cDNA, produced by restriction of pCB6-YY1 with *Eco*RI, was ligated into the *Eco*RI site of the murine stem cell virus-long terminal repeat (MSCV-LTR) retroviral vector pKMV upstream of an internal ribosome entry site (IRES) and enhanced green fluorescence protein (EGFP). The resultant construct YY1-pKMV, or pKMV alone, was transfected into the Phoenix ecotropic packaging cell line using calcium phosphate and cultured in DMEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). Supernatant containing replication-defective virus was collected 2 days post transfection and used to transduce (www.stanford.edu/group/nolan/protocols/pro_helper_dep.html) the rat mammary adenocarcinoma 13762 MAT cell line (8) maintained in RPMI-1640 containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). Two days post-infection, $EGFP^+$ cells were sorted by flow cytometry and used in primary tumor growth experiments. Female Fischer 344 rats (10-13 w.o.) were injected subcutaneously with $1x10^6$ 13762 MAT cells transduced previously with YY1-pKMV or pKMV alone in 100 μl of RPMI-1640 containing 10% FBS. After 21 days, the rats were sacrificed and primary tumors removed, weighed and fixed in formalin. Microvascular density was evaluated by blinded microscopic examination of 6 entire H&E-stained cross-sections under high power field each from 3 tumors per group. Five animals were used in each group, and the experiment was performed twice.

Immunohistochemical staining. Staining was performed with antibodies on consecutive paraffin sections of formalin-fixed tissues. Prior to staining, deparaffinized sections were boiled in citrate buffer, pH 6.0, to retrieve antigenicity, and treated with 3% hydrogen peroxide. After washing with PBS, pH 7.4, sections were incubated with primary antibody for 1 h, followed by incubation with the secondary antibody (goat anti-rabbit; BA-1000: Vector) or horse anti-mouse BA-2000 for 1 h and finally with avidin-biotin complex (Elite ABC kit; PK-6100, Vector). Bound avidin-biotin complexes were visualized by treatment with 3,3'-diaminobenzidine (DAB) solution for 2 min, which produced brown coloration. Sections were counterstained with Mayer's hematoxylin.

Immunoprecipitation analysis. Cells were transfected with indicated constructs, and after 24 h, harvested in 1X RIPA buffer and precleared with prewashed protein G-sepharose beads for 1 h prior to incubation with the indicated primary antibody for 1 h at 4˚C with gentle shaking. Pre-washed sepharose beads were incubated with the lysate/antibody mixture for a further 2 h. Beads were washed several times with 1X RIPA followed by a final wash with 200 mM NaCl/RIPA. Proteins were resolved by 12.5% SDS-PAGE and immunodetected by Western blot analysis.

Animal ethics and statistics. Animal experiments were conducted with the approval of the Animal Ethics Committee of John Curtin School of Medical Research, Australian National University (Canberra, Australia). Values are expressed as the mean + SEM. Differences between groups were tested for statistical significance using ANOVA as indicated in the text and considered significant at $p<0.05$.

Results

YY1 suppressed tumor cell growth. We introduced exogenous YY1 into MCF7 human breast carcinoma cells in culture using either a CMV-based plasmid (pCB6+) or by adenovirus (pAd-Easy). Exposure of growth-quiescent MCF7 cells to medium containing serum increased cell proliferation within 3 days (Fig. 1). YY1 significantly inhibited MCF7 proliferation, following delivery by either plasmid (Fig. 1, left) or adenovirus (Fig. 1, right). In contrast, neither transfection with equivalent amounts of plasmid backbone, nor transduction with the LacZ adenovirus perturbed MCF7 cell growth (Fig. 1). In support of these data, YY1 overexpression confirmed by Western blotting, reduced levels of PCNA (Fig. 1, upper right), a marker of cell growth. YY1 inhibition of tumor cell growth was not confined to human breast carcinoma cells. Transduction of U178 human glioblastoma cells with YY1 resulted in significant, dose-dependent inhibition of serum-inducible tumor cell growth within 3 days (Fig. 2, left). Western blotting further confirmed that YY1 was overexpressed in the transduced cells (Fig. 2, right).

YY1 suppressed 13762 MAT tumor growth in rats without influencing tumor microvascular density. To evaluate the influence of YY1 on tumor growth in an animal model we transduced 13762 MAT rat mammary adenocarcinoma cells with a green fluorescent protein (GFP)-based retroviral construct generating YY1. GFP^+ cells were sorted by flow cytometry and implanted subcutaneously into rats. YY1 inhibited MAT tumor growth by approximately 80% compared with the GFP alone group 21 days after injection (Fig. 3A, right), a growth inhibitory effect that was clearly evident visually (Fig. 3A, upper left). This growth inhibitory effect was not due to YY1 suppression of tumor angiogenesis. Assessment of microvascular density in the resected tumors did not demonstrate a difference between the Retro-GFP-YY1 and Retro-GFP groups despite the significant difference in tumor size (Fig. 3A, right, inset). In support of YY1 suppression of 13762 MAT tumor growth *in vivo*, YY1 significantly inhibited serum-inducible 13762 MAT cell proliferation *in vitro* and did so in a dose-dependent fashion (Fig. 3A, lower left).

YY1 suppressed PCNA expression and pRb phosphorylation in 13762 MAT tumors. pRb phosphorylation inactivates pRb and permits cell cycle progression (9). We therefore investigated whether YY1-dependent growth inhibition in the 13762 MAT tumors caused reduction in levels of pRb phosphorylation. Immunohistochemical staining revealed that YY1 was indeed overexpressed in the Retro-GFP-YY1 tumors. This in turn reduced both $pRb^{Ser249/Thr252}$ expression and resulted in a concomitant reduction in PCNA (Fig. 3B).

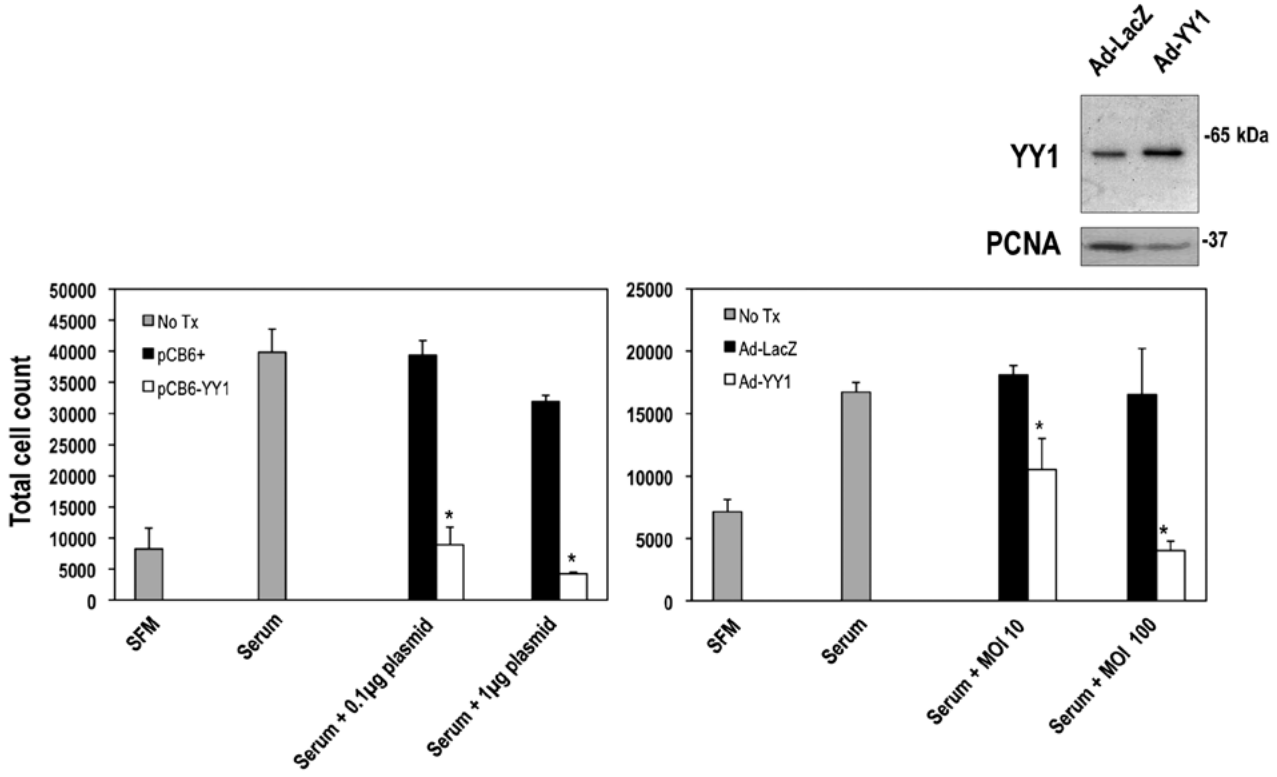

Figure 1. YY1 suppresses MCF7 cell proliferation. MCF7 cells were transfected with the indicated amount of pCB6^{+} or pCB6-YY1 (left panel), or transduced with Ad-LacZ or Ad-YY1 (right panel). After 3 days the cells were trypsinized, resuspended in Isoton II, and quantified using a Coulter counter. No Tx denotes no treatment. *In vitro* proliferation experiments were performed in triplicate. Vertical bars represent ± SEM, $^{*}p<0.05$. Alternatively, YY1 and PCNA protein levels were determined 24 h after transduction with 10 MOI of Ad-YY1 or Ad-LacZ by Western blot analysis (top right panel).

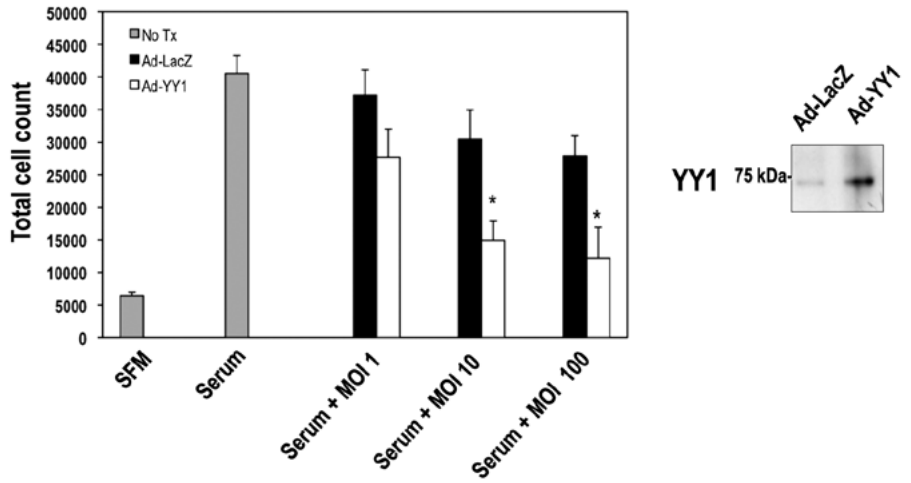

Figure 2. YY1 suppresses U178 cell proliferation. U178 cells were transduced with various amounts of Ad-YY1, and after 3 days the cells were trypsinized, resuspended and quantified (left). No Tx denotes no treatment. SFM denotes serum-free medium. *In vitro* proliferation experiments were performed in triplicate. Vertical bars represent ± SEM, $^{*}p<0.05$. Alternatively, U178 cells were transduced with 10 MOI of Ad-YY1 or Ad-LacZ and after 24 h Western blotting was performed for YY1 expression (right).

YY1 suppressed p21$^{WAF1/Cip1}$ expression and inhibited p21$^{WAF1/Cip1}$ complex formation with cdk4 and cyclin D1. Since Rb phosphorylation lies downstream of a p21$^{WAF1/Cip1}$-cdk4-cyclin D1 cell cycle regulatory complex 6, we determined the effect of YY1 on p21$^{WAF1/Cip1}$ complex formation with cdk4 and cyclin D1. YY1 overexpression, which was confirmed by Western blotting, reduced p21$^{WAF1/Cip1}$ expression in 13762 MAT cells (Fig. 3C). Moreover, immunoprecipitation studies revealed that YY1 reduced intracellular levels of p21$^{WAF1/Cip1}$-cdk4 and p21$^{WAF1/Cip1}$-cyclin D1 complexes (Fig. 3C), presumably via down regulating p21$^{WAF1/Cip1}$ expression. YY1 overexpression did not affect total levels of cyclin D1, cdk4 or β-actin (Fig. 3C). YY1 therefore inhibits tumor growth and pRb phosphorylation, and inhibits formation of a cell cycle regulatory complex.

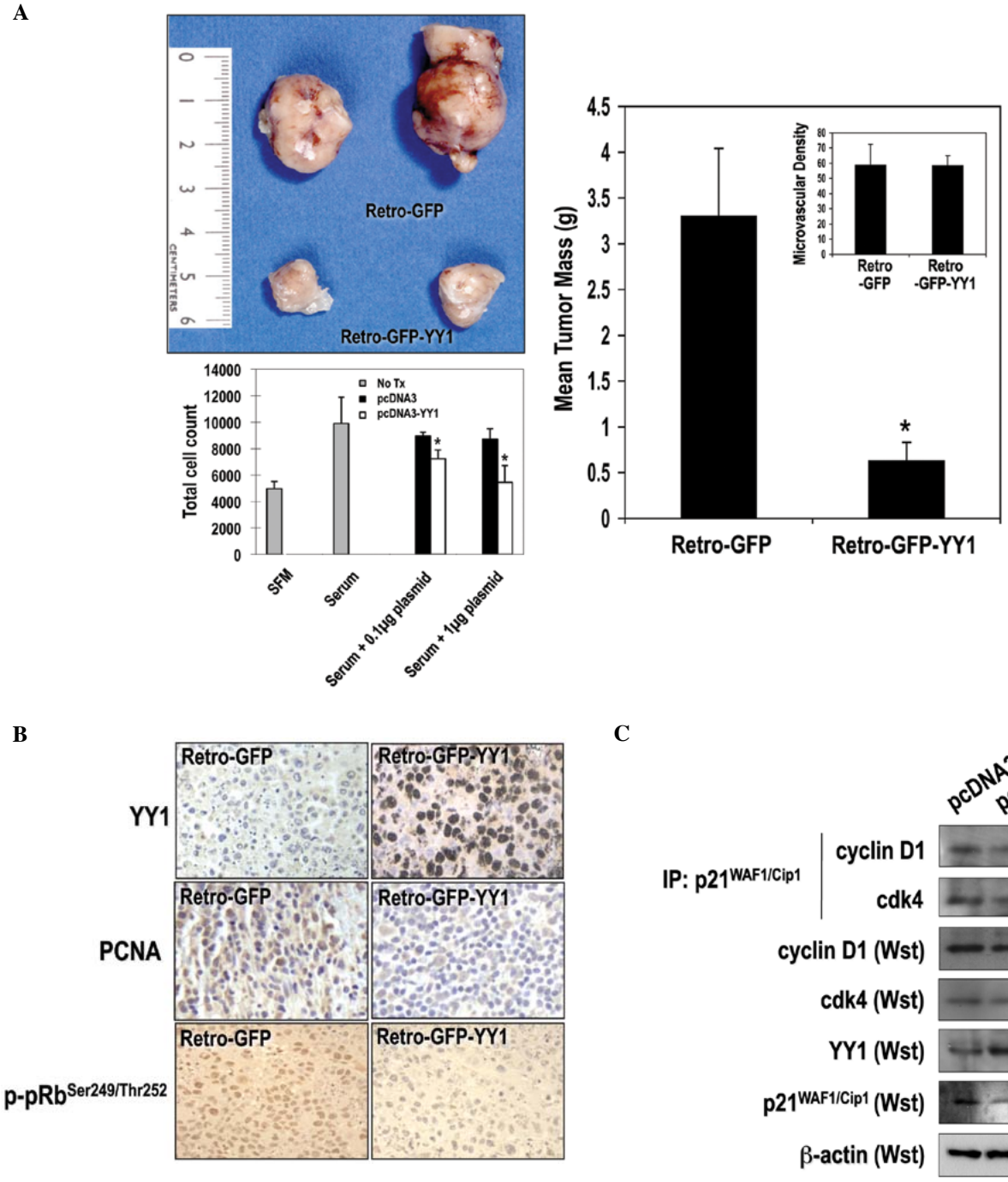

Figure 3. YY1 suppresses 13762 MAT tumor growth in rats without influencing tumor microvascular density. Rat 13762 MAT adenocarcinoma cells were transduced with retroviral-GFP-YY1 (pKMV-YY1) or retroviral-GFP (pKMV) prior to sorting for GFP$^+$ cells by flow cytometry and subcutaneous implantation in rats. (A) Tumors from each cohort were resected after 21 days, weighed and data expressed as mean tumor weight (right). Representative gross images of tumors (upper left). The inset represents blinded quantification of blood vessel density per high power field in the tumors. Alternatively, 13762 MAT cells were transfected *in vitro* with the indicated amounts of pcDNA3 or pcDNA3-YY1. After 2 days the total number of cells in the wells were collected and resuspended in Isoton II, and quantified using a Coulter counter (lower left). No Tx denotes no treatment. *In vitro* proliferation experiments were performed in triplicate. Vertical bars represent ± SEM, $^*p<0.05$. (B) Immunostaining of the 13762 MAT tumors for YY1, PCNA and pRb$^{Ser249/Thr252}$. Antibodies were used at a dilution of 1:200 (YY1, PCNA, p-pRb$^{Ser249/Thr252}$). (C) 13762 MAT cells were transfected with 30 μg pcDNA3 or pcDNA3-YY1 and after 24 h, extracts were subjected either to Western blot (Wst) analysis for cdk4, cyclin D1, YY1 or p21$^{WAF1/Cip1}$, or immunoprecipitation (IP) with p21WAF1/Cip1 antibodies followed by blotting for cdk4 or cyclin D1. SFM, serum-free medium.

Discussion

The role of YY1 in cancer cell growth is controversial. On the one hand, YY1 has been implicated in cell cycle progression, tumor cell resistance to chemotherapeutic agents and in the initiation of tumorigenesis (10-12). For example, YY1 physically interacts with Hdm2 and the tumor suppressor p53, and stimulate Hdm2-mediated p53 polyubiquitination and degradation (13). YY1 also negatively regulates Fas and death receptor-5 (DR5) in several tumor cell types (14). Nitric oxide increases Fas expression via inactivation of YY1 binding to the silencer region of the Fas promoter (14). Moreover

chemotherapeutic drug-induced sensitization of tumor cells to TRAIL-mediated apoptosis (by CDDP, ADR, etoposide, vincristine) involves inhibition of YY1 and up-regulation of DR5 expression (15). Elevated YY1 protein levels are associated with a number of cancer types such as high-grade squamous cervical intraepithelial lesions, cervical cancer and HPV infection of the cervix (16). This has led to the suggestion that YY1 may serve as a diagnostic and prognostic tumor marker, and a drug target in cancer therapy (11).

The present findings support YY1 serving an alternative role, namely acting as a tumor suppressor at least in an overexpression setting. A recent study also demonstrates that YY1 overexpression inhibits breast cancer (MDA-MB-231) xenograft growth in nude mice and suggests that YY1 may serve as a tumor suppressor for breast cancer formation (17). Lee *et al* compared YY1 expression between breast cancer and normal breast tissue and found the majority of normal tissues examined had higher YY1 levels than tumors (17). In certain other tumors such as adenocarcinoma and melanoma, YY1 expression is reduced compared to benign and normal counterparts (18). YY1 is expressed comparatively lower in metastatic malignant melanoma than malignant melanoma, and again in pediatric osteosarcoma versus normal osteoblasts (18). Lastly, while YY1 may be involved in prostate cancer development, decreased YY1 may give metastatic cells a survival advantage (19). Our studies show that YY1 can negatively regulate the growth of multiple malignant cell types.

Our findings suggest that YY1 inhibition of 13762 MAT cell growth involves its suppression of $p21^{WAF1/Cip1}$ expression and inhibition of complex formation between $p21^{WAF1/Cip1}$ and cdk4, and $p21^{WAF1/Cip1}$-cyclin D1. pRb phosphorylation and PCNA are well-established markers of tumor cell proliferation (20,21) and both factors are suppressed by YY1. PCNA was originally identified as an antigen that is expressed in the nuclei of cells during S-phase (22) and is now known to clamp DNA polymerase δ to DNA (23). pRb phosphorylation inactivates this tumor suppressor, releasing E2F and facilitating cell cycle progression (6,24). Alternatively, or even in addition, YY1, as a transcriptional repressor, may inhibit the expression of endogenous growth factors and cytokines required for autocrine tumor cell growth, or even increase the expression of growth inhibitors. For example, YY1 binds to and represses the promoter of EGF (25) which, through its receptor EGF-R, has been widely implicated in tumor progression, invasion and metastasis (26). Other mechanisms may also be at play. YY1 is a potent positive regulator of BRCA1 gene expression, which is generally reduced in sporadic breast cancer (17). In addition, YY1 activates HLJ1, a novel tumor and invasion suppressor that inhibits tumorigenesis and cancer metastasis (27).

Our inability to detect differences in microvascular density between the YY1-transduced and control tumor groups is consistent with our previous demonstration that YY1 does not inhibit vascular endothelial cell growth (5,6). This suggests that YY1 suppression of tumor growth is direct, rather than indirect via inhibition of a vascular supply.

In summary, we show here that YY1 can inhibit the growth of different tumor cell types *in vitro*, and block solid tumor growth in rats, inhibit PCNA expression and reduce $pRb^{Ser249/Thr252}$ phosphorylation. These findings show that YY1 can negatively regulate the growth of multiple malignant cell types.

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