The Melanoma-Associated Antigen A3 Mediates Fibronectin-Controlled Cancer Progression and Metastasis

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Abstract
Tumor cells frequently exhibit decreased adhesiveness due to failure to deposit stromal fibronectin (FN), permitting more rapid proliferation, migration, invasion, and metastasis. Although up-regulation of FN has been noted in gene profiles of carcinomas compared with normal tissue, reduced FN expression has been described at the peripheral margins of invading tumors. In this study, we investigate the role of FN in cancer behavior. Using human thyroid carcinoma cells with stably down-regulated FN, we performed gene profiling and created an orthotopic mouse model. We stably overexpressed the FN target, MAGE A3, which has also been identified as a target of the breast cancer risk factor fibroblast growth factor receptor 2, and examined the functional effects in vitro and in vivo in a flank model and an orthotopic model of thyroid cancer. Mouse xenografts showed significantly enhanced tumor growth as well as larger and more numerous lung metastases in response to FN silencing. Gene profiling identified the melanoma-associated antigen (MAGE A3) as significantly up-regulated in response to FN silencing. Forced expression of MAGE A3 resulted in p21 down-regulation, increased cell cycle progression, increased cell migration rate, and invasion in vitro and in vivo in an orthotopic mouse model where microcomputed tomography confirmed lung metastases that recapitulate the progression of human thyroid cancer. We conclude that MAGE A3 is a functional integrator of diverse signals, including FGFR2 and FN, to modulate cancer progression. [Cancer Res 2008;68(19):8104–12]

Introduction
Thyroid carcinomas have a very wide spectrum of differentiation from some of the most indolent papillary microcarcinomas to the most invasive and lethal anaplastic malignancies (1). This spectrum of progression has been linked with a pattern of cumulative genetic defects that correlate with tumor differentiation, metastatic potential, and aggressiveness (1). Thus, thyroid cancer provides a model to examine the effects of targeted modulation of cell growth and differentiation in human neoplasms of varying degrees of differentiation and with specific genetic defects. Neoplastic transformation is often characterized by changes in the organization of the cytoskeleton, decreased cell adhesion, and aberrant adhesion–mediated signaling (2). Disruption of normal cell adhesion contributes to enhanced proliferation, migration, and invasion leading to metastasis. Fibronectin (FN) is an extracellular matrix protein with putative roles in mediating these actions. Indeed, tumor cells with decreased adhesiveness frequently fail to deposit stromal FN (3). In particular, reduced FN expression has been noted in transformed cell lines and primary tumors (4), including thyroid cancer (3, 5, 6), where diminished FN has been identified at the periphery of invasive tumor margins. In this context, we found that down-regulation of FN stimulates thyroid cancer cell proliferation and tumor growth (7). Conversely, 1, 25-dihydroxy vitamin D₃ treatment increases cell adhesiveness and inhibits cell proliferation and tumor growth through enhanced FN expression (7).

To determine the role of FN in mediating disease progression, we examined the effect of FN down-regulation on cancer metastasis using an orthotopic mouse model of thyroid cancer. We used this system to identify, through gene profiling, novel putative intracellular targets of FN action. The data uncover the melanoma-associated antigen 3 (MAGE A3) as a mediator of FN action, a clinically relevant outcome in the human form of this malignancy. MAGE A3 was also recently identified as a target of fibroblast growth factor receptor (FGFR) 2 signaling (8). As the latter is the same FGFR member linked in genome-wide arrays with increased breast cancer risk (9, 10), the current studies are particularly relevant to establish the integrative role of MAGE A3 in response to diverse signals to modulate cancer progression.

Materials and Methods

Cell culture. The human thyroid follicular WRO carcinoma cell line, originally established by Dr. G. Juillard (University of California at Los Angeles, Los Angeles, CA), was kindly provided by Dr. J. Fagin (Memorial-Sloan Kettering, New York, NY). Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine, 1 mmol/L sodium pyruvate, 1 × nonessential amino acids, and antibiotics. TPC-1 cells, a well-differentiated papillary carcinoma cell line, were cultured in DMEM with 5% serum and 2 mmol/L l-glutamine (11).

Transfections. Cell transfections were performed using Lipofectamine (Invitrogen Corp.). Cells were cultured in 6-cm plates until 60% to 70% confluent and transfected with 5 μg of the human MAGE A3 cDNA in pcDNA3.1 (12). One day after transfection, cells were placed into selection medium containing 1 mg/mL G418 (Invitrogen) and individual geneticin-resistant colonies were screened by reverse transcription-PCR (RT-PCR) and Western blotting. At least two independent stably transfected clones were used for subsequent studies. The target sequence for FN down-regulation, AAC AAA TCT CCT GGC TGG TAC, was aligned to the human genome database in a BLAST search to exclude homology with unrelated genes. The shRNA oligonucleotide templates were as follows: top strand, GAT CCG CAA ATC TCC TGC CTG CTG TCC TGC CTG TGG AGT ACC AGG CAG GAG ATT TGT TTT TTT GAA A; bottom strand, AGC TTT TCC AAA AAA CAA ATC TCC TGC CTG CTG TGC CTG TGG TCTG AGT CAG AGT ACC AGG CAG GAG ATT TGC G. These were prepared according to the manufacturer’s instructions (Ambion, Inc.). After annealing, the duplexes were ligated into pSilencer 2.1-U6 neo vector as described previously (7).
Fibronectin and MAGE A3 in Cancer Progression

Oligonucleotide microarray analysis. Total RNA was extracted from cultured cells using an RNeasy kit (Qiagen, Inc.) as described previously (8). Array hybridization to the Affymetrix human U133Plus2 GeneChip was conducted at The Centre of Applied Genomics (Hospital for Sick Children, Toronto, Canada). RNA from two independent FN-shRNA clones and two independent scrambled control clones were subjected to in vitro transcription, labeling, and hybridization using standard Affymetrix reagents. Hybridized chips were washed, and scanned on an Affymetrix GeneChip 3000 confocal scanner. Raw microarray data were analyzed by ArrayAssist software (lobion) using the PLIER algorithm as described previously (13). Genes were considered to be differentially expressed if the signal changed at least 2-fold (or signal log2 ratio 1). The four data sets (two for FN-shRNA and two for scrambled controls) were analyzed using gene expression and statistical tools in Spotfire's DecisionSite software package. To identify gene targets that had significantly altered gene expression, FN-shRNA transfected clones versus scrambled control groups were compared with each other using the Student’s t test as described previously (13).

RNA extraction and RT-PCR analysis. Total RNA was isolated from cultured cells using an RNeasy kit (Qiagen, Inc.) followed by DNase I treatment. cDNA was generated using the TaqMan Reverse Transcription Reagent kit (Applied Biosystems). Primers-prIME A3/6 and phosphoglycerate kinase (PGK)-1 (as an internal control) were previously described (8). Amplification was performed using HotStarTag DNA polymerase kit (Qiagen). PCR conditions were as follows: (a) 95°C for 15 min; (b) 30 cycles of 94°C for 30 s, 56 or 58°C for 30 s, and 72°C for 1 min; (c) 72°C for 10 min; and (d) 4°C hold. Negative controls omitting reverse transcriptase and positive controls were included in each PCR reaction (data not shown).

Protein extraction and Western blotting. Cells were lysed in radioimmunoprecipitation assay buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/mL phenylmethylsulfonyl fluoride, aprotinin, and sodium orthovanadate in PBS). Total cell lysates were incubated on ice for 30 min followed by microcentrifugation at 10,000 × g for 10 min at 4°C. Fifty micrograms of protein were separated by 10% or 12% SDS-polyacrylamide electrophoresis and transferred onto nitrocellulose membranes, which were blocked in 5% nonfat milk and 0.1% Tween 20 in TBS (20 mmol/L Tris-Ci and 500 mmol/L NaCl (pH 7.5)) for 1 h and incubated overnight at 4°C with the following primary antibodies: MAGE (6C1; 1:200; Santa Cruz Biotechnology, Inc.), p21 (1:500; BD Biosciences), p27 (1:1,000; Transduction Laboratories), phospho-Rb (Ser807/811; 1:1,000; Cell Signaling Technology), human p53 (DO-1; 1:1,000; Santa Cruz Biotechnology, Inc.), mouse p53 (1:1,000; Chemicon International), and actin (1:500; Sigma). After washing, membranes were incubated for 1 h at room temperature with peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody (1:2,000; Santa Cruz). Protein bands were visualized by chemiluminescence as recommended by the manufacturer (Amer sham). Cell cycle analysis. For cell cycle assessment, cells were starved in serum-free growth medium for 24 h before being exposed to growth medium containing 10% FBS for another 24 h. After trypsinization, cells were washed with D-PBS (Ca2+–Mg2+ free) and fixed with cold 30% ethanol for 1 h on ice. Fixed cells were washed with staining buffer (0.2% Triton X-100 and 1 mmol/L/1 EDTA (pH 8.0), in PBS) and resuspended in the staining buffer containing 50 µg/mL DNPase-free RNase A (Sigma) and 50 µg/mL propidium iodide for 1 h. Cell cycle analysis was performed by fluorescence-activated cell sorting (Becton Dickinson) using CellQuest analysis and specific S phase was analyzed using ModFit DNA Analysis (Verity Software House, Inc.). Migration assay. Migration assays were performed as described previously (14). Control and MAGE A3–transfected cells were plated on collagen IV–coated 24-well plates (BD Biosciences). After reaching confluence, cells were gently scratched using a standard 200 µL pipet, rinsed three times, and allowed to migrate on a collagen IV surface. Serial photographs were obtained every 10 min for 20 h using an Axiosvert 200 M phase-contrast microscope. A video was constructed from the individual frames taken every 20 min for 20 h. Data from experiments performed in duplicate are expressed as the mean ± SE from three independent experiments.

Invasion assay. Invasion assay was performed as described previously (14). Briefly, BioCoat Matrigel Invasion Chambers (BD Biosciences) were pretreated by the manufacturer. After trypsinization, 2 × 105 cells suspended in 0.5 mL of serum-free RPMI1640 medium containing 0.2% bovine serum albumin were plated in each insert. After 24 h of incubation, noninvading cells were removed from the upper surface of the membrane by scrubbing with a cotton swab. Cells on the lower surface of the membrane were stained with Diff-Quick Stain (BD Biosciences). After air drying, membranes were removed from the insert; invading cells in the whole membrane were photographed using a Slide Scanner and analyzed for quantification.

Xenografted mouse models of thyroid cancer. WRO cells (5 × 106) forced to express MAGE A3 and their empty vector control were xenografted in the flanks or orthotopically implanted in the neck of 5 to 6-wk-old severe combined immunodeficient (SCID) mice for assessment of tumor growth and metastasis. Animal handling and treatment protocols were approved by the Ontario Cancer Institute Animal Care and Utilization Committee. Tumor growth and volume were measured using calipers every 5 d (tumor volume in mm3 = tumor width × tumor length × tumor length/2).

Results

Down-regulation of FN promotes lung metastasis of thyroid cancer. Established stable lines of FN-deficient human thyroid carcinoma WRO cells have been previously described (7). This down-regulation of FN stimulates thyroid tumor growth in flank xenografts (7). To specifically examine the effect of FN on metastasis development, we used an orthotopic mouse model of thyroid cancer (15). This approach confirmed that down-regulation of FN promotes tumor growth in situ (Fig. 1A) and also showed that loss of FN is associated with a significantly increased number and size of lung metastasis (Fig. 1B). In particular, only 1 of 9 control mice developed a single small metastatic lung lesion. In contrast, 6 of 9 mice injected with cells stably transfected with FN
shRNA developed multiple lung metastases \( (P < 0.05) \), which were also significantly larger in size (Fig. 1B).

**Down-regulation of FN increases MAGE A gene expression.** To investigate the specific targets of FN, we performed cDNA microarray profiling comparing human thyroid cancer WRO cells expressing FN si-RNA or scrambled sequence. The majority of spotted genes were not affected by FN down-regulation. However, FN down-regulation increased the expression of MAGE A family genes (Table 1). Comparison of these cells by RT-PCR and by Western blotting confirmed an increase of MAGE A3 expression (Fig. 2A). Similarly, immunohistochemical examination of cell pellets and corresponding xenografted mouse tumor tissues confirmed MAGE A3 up-regulation in response to FN shRNA (Fig. 2B).

**MAGE A3 stimulates thyroid cancer cell proliferation in vitro.** To examine the functional role of MAGE A3, we forced its stable expression in WRO cells (Fig. 3A). Using flow cytometry, we found that MAGE A3 significantly reduces the proportion at G0-G1 phase and increases entry into S phase (Fig. 3B). Consistent with this finding, MAGE A3 decreased p21 levels, but not p27, to promote Rb phosphorylation (Fig. 3C).

**MAGE A3 down-regulates p21.** MAGE proteins can function as cofactors supporting KAP1-dependent suppression of p53 action (16, 17). To better understand the mechanism underlying MAGE A3 action, we tested the effect of this cancer antigen on p21 as a target of p53 action. In WRO cells, which harbor a heterozygous p53 mutation, forced MAGE A3 expression resulted in diminished p21 levels (Fig. 3D). The same effect was also seen in MAGE A3-transfected human TPC-1 thyroid carcinoma cells that harbor wild-type p53 (Fig. 3D). These findings suggest that MAGE A3 can down-regulate p21 in the presence of wild-type or mutant p53.

**MAGE A3 promotes cell migration and invasion in vitro.** To further investigate the functional effect of MAGE A3 expression, we examined the ability of MAGE A3 to influence cell migration on a collagen IV matrix. We found that MAGE A3 significantly promotes cell migration as determined by dynamic video monitoring (Supplementary videos 1 and 2) and illustrated in static images (Fig. 4A) and calculated migration rate (Fig. 4B).

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**Figure 1.** Down-regulation of FN promotes thyroid cancer growth and metastasis. Human-derived thyroid follicular carcinoma WRO cells stably expressing FN-shRNA or scrambled sequence as control were orthotopically introduced into the necks of 6-wk-old SCID mice. A, the indicated tumor volumes represent the mean + SE of nine animals in each group. \(*, P < 0.05\) by paired t test versus control. Significant differences were reached by 10 d after injection and were sustained for the duration of 26-d experiments. B, down-regulation of FN enhances thyroid cancer lung metastasis. The lung of a control mouse contains a single small metastatic lesion (arrow), whereas mice injected with cells expressing FN-shRNA developed multiple larger metastatic lesions (arrows).
To determine the effect of MAGE A3 on cell invasion, we examined independent clones of WRO cells with forced MAGE A3 expression and their controls using a Matrigel invasion assay. These studies showed that MAGE A3 significantly promotes cell invasion (Fig. 4C).

MAGE A3 promotes thyroid tumor growth and lung metastasis in vivo. To study the effect of MAGE A3 on tumor growth in vivo, we used a thyroid cancer flank xenograft mouse model as previously described (7). This showed that tumor volume was significantly increased in response to forced MAGE A3 expression (Fig. 5A). Furthermore, using an orthotopic model, we found that MAGE A3 significantly promotes tumor growth. Specifically, 14 of 17 animals in the MAGE A3 group developed metastases (Fig. 5B) compared with 3 of 20 in the control pcDNA group (P < 0.001). The development of pulmonary metastases was monitored in vivo by micro-CT imaging (Fig. 5C) that showed more numerous and larger metastatic foci in animals bearing tumors that expressed MAGE A3. Histopathologic examination confirmed more numerous and larger lung metastases in the MAGE A3 group (Fig. 5D, left) compared with control animals that had minimal lung involvement. Furthermore, we identified selective strong MAGE A3 immunostaining in metastatic tumor deposits (Fig. 5D, right) providing additional evidence for the involvement of this oncogene in metastasis formation.

Discussion

The present findings along with our previous work (7) support an important role for FN in cancer progression. We show that down-regulation of FN stimulates thyroid cancer cell proliferation, accelerates cell migration and invasion, and promotes neoplastic growth in vivo. More strikingly, using an orthotopic mouse model of thyroid cancer, we show that the loss of FN significantly enhances lung metastasis, a feature that closely mimics an important element of the human disease. These findings are consistent with a model wherein FN acts as a tumor suppressor to

Table 1. Microarray analysis of FN target genes

<table>
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<th>Gene Symbol</th>
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<td>Ratio 1</td>
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<tr>
<td>MAGE A6</td>
<td>Melanoma antigen family A, 6</td>
<td>214612_x_at</td>
<td>3.88</td>
</tr>
</tbody>
</table>

NOTE: Ratio 1 indicates the fold of expression for each gene as determined by microarray analysis of control shRNA/WRO clone #1 vs FN shRNA/WRO clone #1. Ratio 2 indicates the fold of expression of control shRNA/WRO clone #1 vs FN shRNA/WRO clone #2. Ratio 3 indicates the fold of expression control shRNA/WRO clone #2 vs FN shRNA/WRO clone #1. Ratio 4 indicates the fold of expression of control shRNA/WRO clone #2 vs FN shRNA/WRO clone #2.

Figure 2. Down-regulation of FN enhances MAGE A3 expression in thyroid cancer cells. A, RT-PCR examination of WRO cells expressing FN shRNA confirms increased MAGE A3/6 mRNA (left) and protein levels by Western blotting (right) compared with control cells expressing a scrambled sequence. PGK-1 serves as a control for RNA stability and actin provides evidence of equal protein loading. Each lane represents an independently transfected stable clone. B, immunohistochemistry of these cells identified strong nuclear reactivity and a pale cytoplasmic blush in vitro (left) and in vivo in xenografted cells (right).
Impede tumor progression and metastasis. FN staining has been reported to be reduced or diminished in many solid neoplasms (4) including thyroid cancer, where diminished stromal FN expression has been noted at the periphery of invasive tumor margins (3). Conversely, up-regulation of FN has been identified in gene profiles of thyroid carcinomas compared with normal tissues (18, 19). These findings suggest that up-regulation of FN in well-differentiated carcinomas may represent a compensatory response to neoplastic transformation. In contrast, dedifferentiated tumors may reach a point of diminished capacity to lay down FN within the stroma. Such a model would explain the remarkable biological difference between well-differentiated and dedifferentiated thyroid carcinomas.

In the present study, we used stable shRNA-mediated FN downregulation to identify targets of its action in thyroid cancer. Using cDNA microarray profiling, we uncovered significant increases of

Figure 3. MAGE A3 promotes cell cycle progression. A, establishment of forced expression of MAGE A3 in endogenously-deficient WRO cells was confirmed in multiple stable clones by RT-PCR (left) and Western blotting (right). PGK-1 serves as a control for RNA stability and actin provides evidence of equal protein loading. B, forced MAGE A3 significantly stimulates cell cycle progression. Synchronized cells were examined by flow cytometry. Note the reduced residence of MAGE A3–expressing cells in G0-G1 with increased proportion in S phase. C, forced MAGE A3 expression diminishes p21 (shown by Western blot and immunohistochemistry with nuclear localization), but not p27, and increases Rb phosphorylation (pRb). Actin provides evidence of equal loading. D, forced MAGE-A3 diminishes p21 levels in p53 mutant WRO cells (left) and in TPC-1 cells with wild-type p53 (right). Each lane represents an independent stably transfected clone.
MAGE A3 levels in the face of loss of FN. This melanoma-associated antigen is a member of a group of >50 closely related peptides (20) classified into 2 major subgroups based on their gene structure and expression profiles (21). Subgroup 2 MAGE members are expressed in various normal adult human tissues (21). In contrast, subgroup 1 members, including MAGE A, B, and C, are expressed only in normal testis, placenta, and certain stages of embryonal development (21, 22). However, MAGE A members have been identified in a variety of malignant tumors (23). MAGE proteins are also called cancer-testis antigens (24) as they are recognized by autologous CTLs in a MHC class I-restricted fashion (25). Based on their immunogenicity in cancer, MAGE or cancer-testis antigens represent ideal targets for cancer vaccines (26). Indeed, immunotherapeutic trials targeting MAGE peptides have achieved encouraging results in patients with metastatic melanoma (27–29).

Figure 4. Forced expression of MAGE A3 significantly enhances cell migration and invasiveness. A, MAGE A3–expressing and control WRO/pcDNA cells were plated and allowed to migrate on collagen IV surface after scratching a wound in a monolayer of cells. Serial photos were taken every 10 min for 20 h using an Axiocamt 200 M phase-contrast microscope at >5 magnification. The video was composed of individual frames taken every 20 min for 20 h. To obtain better contrast, the Edge Detection Filter was used. Photos were selected at 0, 5, 10, 15, and 20 h for image analysis using Image-Pro Plus Software. B, the migrating rate was determined by measuring cell migrating areas on collagen IV–coated 24-well plates. Data from experiments performed in duplicate are expressed as the mean ± SE obtained in three independent experiments. *, a significant difference from control cells. C, WRO cells stably expressing MAGE A3 and control WRO/pcDNA cells were allowed to invade through Matrigel (left) as detailed under Materials and Methods. Columns, mean from three independent experiments each performed in triplicate; bars, SE (right). *, P < 0.05.
MAGE was first reported to encode a tumor-specific antigen in melanoma cells (25). It is expressed in esophageal (30), breast (31), lung (32), bladder (33), gastric (34), and leukemic cells (35). Recently, MAGE antigens have also been reported in human thyroid carcinomas (36) where immunodetection suggested involvement at an early stage in disease progression. However, there is currently limited functional evidence implicating MAGE A3 action in cancer progression.

In the present study, we investigated the functional role of MAGE A3 using gain- and loss-of-function approaches. Forced MAGE A3 in human thyroid carcinoma-derived WRO cells, which are endogenously deficient of MAGE A, resulted in accelerated cell cycle progression with diminished G0-G1 residence and enhanced S phase entry. These cell cycle changes were accompanied by reduced p21 levels and enhanced Rb phosphorylation. Indeed, MAGE A3 significantly diminished p21 levels in TPC-1 cells with
intact p53 and in WRO cells, which are heterozygous for a p53 mutation. Although recent reports implicate MAGE A members in modulating p53 function (16, 17), our data suggest the possibility of p53-independent action of MAGE A3 in targeting p21.

Using a mouse flank xenograft model, forced expression of MAGE A3 significantly stimulated tumor growth. These findings are consistent with one other report where a pan MAGE A shRNA inhibited human melanoma cell proliferation and diminished growth in mouse xenografts (17). Our study shows, for the first time, that selective MAGE A3 expression significantly promotes tumor cell migration and enhances invasive cancer cell growth. More significantly, these effects were accompanied by increased tumor metastasis as shown in a mouse orthotopic model of thyroid cancer. Furthermore, the resulting metastatic deposits were larger and more numerous in the face of forced MAGE A3 expression. Our findings provide a plausible mechanism for the observed effect of MAGE A immunoreactivity correlation with lymphatic vessel invasion in ductal breast carcinoma (37) and with lymph node metastasis in gastric carcinoma (38). However, it must be emphasized that other MAGE A genes members, such as MAGE A4, have been reported to promote apoptosis in non–small cell lung cancer (39). In addition, MAGE D1 may represent a novel endogenous inhibitor of angiogenesis in vitro and in vivo (40). These pleiotropic functions highlight the importance of detailed MAGE characterization to better understand the putative actions of the various family members in cancer progression.

The mechanisms of FN-induced MAGE A3 induction requires further examination. It is clear that MAGE A gene family members are regulated through epigenetic mechanisms including DNA and histone modifications (41). FN is an extracellular matrix protein, which plays a key role in the regulation of cell adhesion, migration, invasion, and metastasis formation. It can be envisioned that FN down-regulation might force cells to lose adhesiveness and enhance their migratory and invasive properties. Furthermore, the pivotal role of MAGE A3 in thyroid cancer progression was underscored through its recent identification as a signaling target of FGFR2 (42). Indeed, FGFR7-mediated activation of FGFR2 results in down-regulation of MAGE A3 through enhanced histone 3 K9 methylation (8). Given the recent linkage of FGFR2 in genome-wide studies with increased breast cancer risk (9, 10), the current elucidation of the role of MAGE A3 in integrating diverse signals will also prove critical to clarify breast cancer progression.

In summary, this study shows that FN can act as a tumor suppressor and an inhibitor of cell proliferation and metastasis. Using combined in vitro and in vivo models, we show that down-regulation of this adhesion factor significantly enhances MAGE A3 expression. MAGE A3, as a down-stream target of FN, accelerates cell cycle progression through p21 reduction, an effect facilitated by diminished p53 levels. Taken together, our findings highlight the importance of MAGE A3 in cancer progression and provide a strong rationale for this tumor-associated antigen as a potential diagnostic and immunotherapeutic target in thyroid cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Received 6/4/2008; revised 7/11/2008; accepted 7/21/2008.
Grant support: Canadian Institutes of Health Research/CBCRA (MOP-86493), the Toronto Medical Laboratories, and the Rita Banach Thyroid Cancer Research Fund.

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We thank the Spatio-Temporal Targeting and Amplification of Radiation Response program and its affiliated funding agencies.

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