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Suppression of α5 gene expression is closely related to the tumorigenic properties of uveal melanoma cell lines

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Summary

Cancer aggressiveness is related to the ability of cancer cells to escape the anchorage dependency toward the extracellular matrix, a process regulated by the integrin α5β1 and its ligand fibronectin. Here, we characterized the expression of the α5 gene in human uveal melanoma cell lines with distinct tumorigenic properties and investigated some of the mechanisms underlying the variations of their malignancy. Strong and weak expression of α5 was observed in cells with no (T108/T115) and high (T97/T98) tumorigenic properties, respectively. Expression and DNA binding of the transcription factors Sp1, activator protein 1 (AP-1) (both acting as activators), and nuclear factor I (NFI) (a strong repressor) to the α5 promoter were demonstrated in all cell lines. A reduced expression of AP-1 combined with a dramatic increase in NFI correlated with the suppression of α5 expression in T97 and T98 cells. Restoring α5 expression in T97 cells entirely abolished their tumorigenicity in immunodeficient mice. These uveal melanoma cell lines might therefore prove particularly useful as cellular models to investigate α5β1 function in the pathogenesis of invasive uveal melanoma.

Introduction

Uveal melanoma is the most common type of primary intraocular tumor in the adult population (Zimmerman, 1965), its incidence reaching 4.3 cases per million individuals in the United States (Singh and Topham, 2003). Unlike skin melanoma, which metastasize primarily to the lymph nodes and the lungs, uveal melanoma will propagate to the liver as the first metastatic site in 68% of individuals and then to the lung (Rietschel et al.,

Significance

Uveal melanoma is the most common type of primary intraocular tumor in the adult population. Unlike melanoma from the skin, uveal melanoma will propagate primarily to the liver. Aggressiveness of many types of cancers, including melanomas, has been related to their ability to interact with the many components of the extracellular matrix (ECM), a process that is dependent on the presence of a large family of membrane-bound receptors collectively called integrins. By exploiting a new array of uveal melanoma cell lines, we show that abnormal expression of the α5 integrin subunit gene might play a pivotal function in the tumorigenic properties of this type of cancer and that the transcription factors (TFs) Sp1, nuclear factor I (NFI), and activator protein 1 (AP-1) may each contribute to various levels in the disregulation of that gene.

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2005). Although progression of the liver metastases might take several years before these secondary tumors can be diagnosed, once this organ is invaded, survival becomes a matter of months for the patient (Singh and Borden, 2005). Even though tremendous progress has been achieved in the identification of prognostic markers associated with metastasis (reviewed in Landreville et al., 2008), yet the exact molecular events involved in the hematogenous invasion of uveal melanoma still remain obscure.

As in other types of cancers, the invasion process of uveal melanoma requires loss of anchorage-dependent growth, degradation of the ECM, and movement of the cell body (Stetler-Stevenson, 2001; Yu et al., 1996). Attachment of the cells to the ECM is essentially mediated by membrane-bound receptors that belong to the integrin family (Hynes, 1992; Van Der Flier and Sonnenberg, 2001). To date, attempts to establish a clear correlation between the pattern of integrins expression and the level of aggressiveness of any given type of cancer have proved to be a challenging task as results are often contradictory. Indeed, many studies reported an increase in the level of expression of integrins in different types of cancer cells (Albelda, 1993; Albelda et al., 1990; Felding-Habermann et al., 1992; Schon et al., 1996), including melanoma cell lines (Gehlsen et al., 1992; Nip et al., 1992), also reviewed in (Mizejewski, 1999). On the contrary, others reported that aggressiveness of some cancer types was inversely related to the level to which certain integrins, primarily those from the β1 subfamily, are expressed in these cancer cells (Damjanovich et al., 1992; Koretz et al., 1991; Nigam et al., 1993; Peltonen et al., 1989; Pignatelli et al., 1990, 1991; Zutter et al., 1990). In spite of this, reduced expression of the fibronectin (FN)-binding integrin $\alpha5\beta1$ remains frequently associated with the transformed phenotype in many human and murine tumors (Stallmach et al., 1992; Weinel et al., 1992; Zutter et al., 1993).

As they bridge the cell cytoskeleton to the ECM, integrins also play a pivotal role in informing the cell from any change occurring in its environment. Their influence is transmitted to the nucleus through the activation of a few signal transduction pathways and causes the post-translational modification of a number of TFs, including Sp1 (Larouche et al., 2000), AP-1 (Troussard et al., 1999), and NFI (Gingras et al., 2009). Interestingly, basal transcription of the $\alpha5$ gene was shown to be ensured by these three TFs (Gingras et al., 2009). Although both Sp1 and AP-1 are known to positively influence gene transcription, the scenario is far more complex for NFI as the members from this family are as efficient as repressors (Nakamura et al., 2001) than activators (Gao et al., 1996) of gene transcription.

In this study, we characterized four new uveal melanoma cell lines (T97, T98, T108, and T115) that exhibit very distinct tumorigenic properties. Examination of their level of $\alpha5$ integrin subunit at both the protein and mRNA level highlighted a reverse relationship between $\alpha5$ expression and the ability of these cells to produce tumors in vivo. Furthermore, through the characterization of critical TFs (Ap-1, Sp1, Sp3, and NFI), we investigated some of the mechanisms dictating the regulation of $\alpha5$ gene expression and therefore malignancy of these cell lines.

Results

Characterization of the morphological, growth, and invasive properties of uveal melanoma cell lines

To investigate the role of the $\alpha5\beta1$ integrin in the tumorigenic properties of uveal melanoma, four cell lines (T97, T98, T108, and T115) were derived by culturing the primary tumor from individuals diagnosed with this type of cancer (Table S1). Unlike the typical spindle-like morphology of uveal melanocytes (UVM), T97, T98, T108, and T115 cells all appear as mixed spindle/epithelioid culture types (Figure 1A). In addition, they all express the protein markers S100 and MART-1 (no positive staining was observed using the HMB-45 Ab) expressed by human melanomas establishing the melanocytic origin of these cells (Figure S1A; also refer to supporting methods S1). Typical of malignant cells, T97, T98, T108, and T115 had estimated doubling times of 17, 17, 14, and 33 h, respectively, whereas that of UVM was found to be over 78 h (Beliveau et al., 2000). The ability of each cell line to grow in an anchorage-independent manner was next monitored; while UVM failed to grow in soft agar, all other cell lines grew as large colonies after 8 days in soft agar (results are presented for T97 and T108 cells in Figure S1B; also refer to supporting methods S1).

To evaluate the ability of these cells to spontaneously generate tumors in vivo, T97, T98, T108, and T115 cells were injected into immunodeficient CD1-nude mice and tumor formation was monitored over a 30-day period. Both the T97 and T98 cell lines were by far the most tumorigenic as all injected mice developed detectable tumors (with an average tumor size of 226 and 127 mm^3 for T97 and T98 cells, respectively; Figure 1B). Mice injected with T108 cells yielded small yet detectable tumors in three of eight animals (average tumor size: 20 mm^3), whereas only one mouse developed a single, barely detectable tumor when injected with T115 cells (tumor size: 2 mm^3). Immunohistochemical analysis of the tumor tissues produced by T97 cells indicated that nearly all cells stained positive for the melanoma markers HMB45, MART-1, and S100, therefore, establishing their melanocytic origin (Figure 1C). Typical of aggressive uveal melanoma (Maniotis et al., 1999), vasculogenic mimicry patterns could be observed throughout the entire tumors yielded by T97 cells (Figure 1D).

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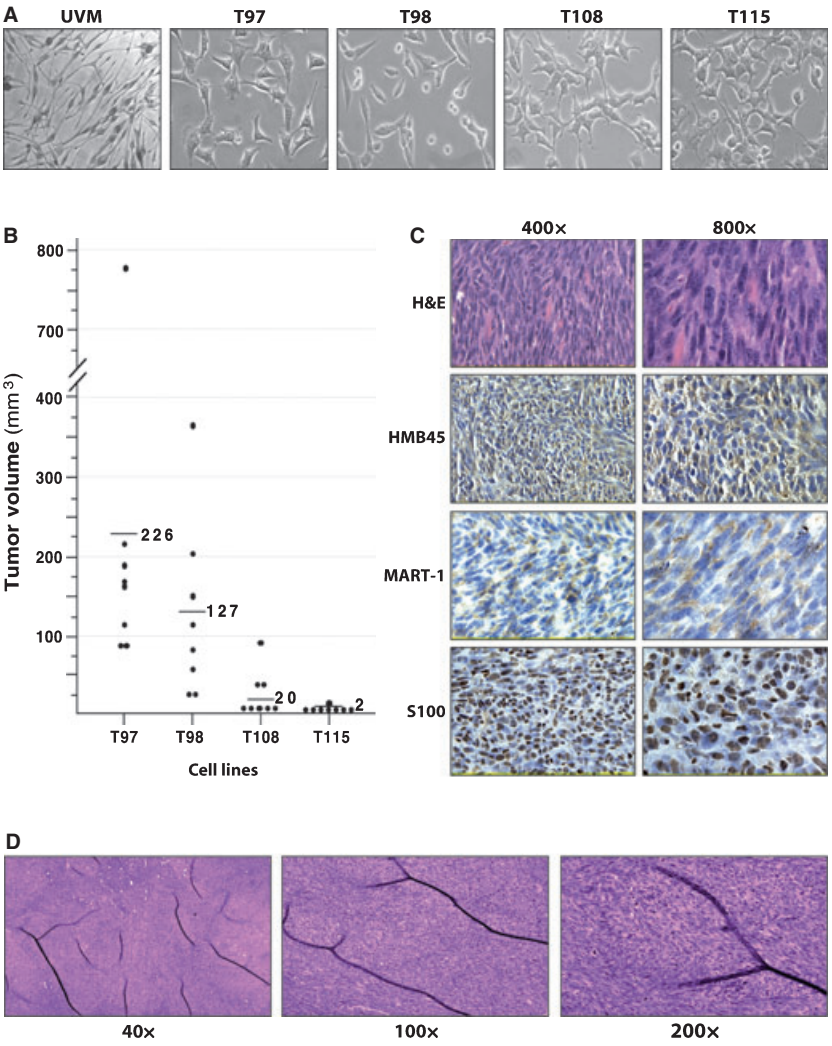

Figure 1. In vivo tumorigenic properties of uveal melanoma cell lines. (A) Phase contrast micrographs of monolayer cultures of either uveal melanocytes or T97, T98, T108, and T115 human uveal melanoma cell lines. Magnification: 200×. (B) Histogram that gives the tumor volume (mm^3) for each of the nude mice injected subcutaneously with the T97, T98, T108, and T115 cell lines. The horizontal bars indicate the mean tumor volumes. (C) Expression of HMB45, S100, and MART-1 by immunohistochemistry on one of the in vivo tumors yielded by the T97 cells. (D) T97 tumor sections stained with hematoxylin/eosin (magnification: 40×, 100×, and 200×).

Expression of α5 in uveal melanoma cell lines is inversely related to tumorigenicity

As alterations in the expression of α5β1 have been reported to be intimately related to the growth properties of many types of cancer cells, we next established whether changes in the pattern of expression for this integrin could correlate with the ability of these cell lines to yield tumors in nude mice. Cell surface expression of both the α5 and α6 (used as a control) subunits was, therefore, monitored in all uveal melanoma cell lines by flow cytometry. As shown in Figure 2A and Table S3, expression of α5 was either entirely absent or very low in the most aggressive cell lines (T97 and T98) whereas it was highly expressed in the cell lines (T108 and T115) that yielded either no or only a few tumors in nude mice (Figure 1B). On the other hand, expression of α6 remained stable between all cell lines (Figure 2A and Table S3). Consistent with these results, RT-PCR analyses indicated that expression of the α6 subunit was quite uniform between all cell lines upon normalization to the 18S transcript (Figure 2B). However, a dramatic reduction in the expression of the α5 transcript was noted in both T97 and T98 cells relative to the level expressed by T108 and T115 cells.

We next determined whether a differential transcriptional activity directed by the α5 gene promoter could correlate with the variations in α5 expression observed between the cell lines with low (T108 and T115) and high (T97 and T98) tumorigenic properties. The plasmid -954α5-CAT that bears the entire α5 promoter up to position -954 relative to the α5 mRNA start site yielded high levels of CAT activity when transfected into T108 and T115 cells but not in T97 and T98 cells (levels 5- to 12-fold lower than in T108 cells were observed; Figure 2C). The reduced promoter activity in T97 and T98 cells was even more drastic when the plasmids -92α5-CAT and -132α5-CAT that only contain the basal α5 gene promoter were used in transfections (Figure 2D). The basal promoter of the α5 gene contained on the -92α5-CAT construct has been shown to bear two closely located target sites for the positive TF Sp1 (Gingras et al., 2003). Mutating each single Sp1 site

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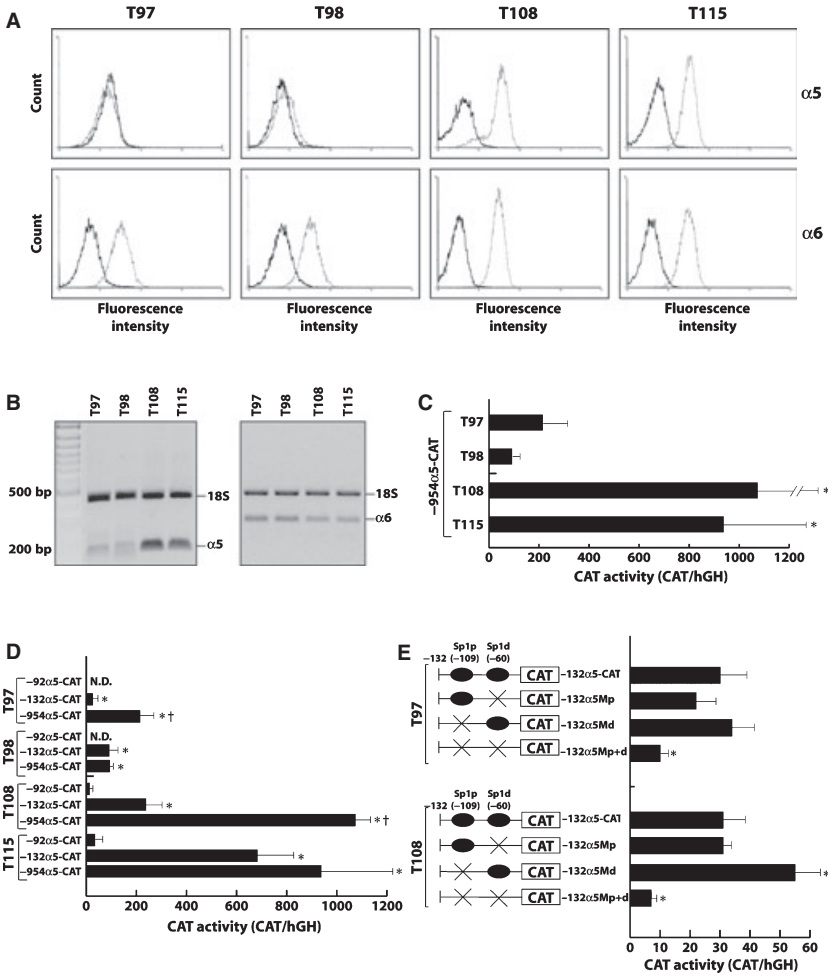

Figure 2. α5 expression and promoter analyses in uveal melanoma cell lines. (A) Expression of the α5 and α6 integrin subunits was monitored by flow cytometry in T97, T98, T108, and T115 cells (thin lines). Thick lines: negative controls. (B) RT-PCR analysis of the α5 and α6 transcripts in T97, T98, T108, and T115 cells. The position of the amplified 222 bp α5 (α5), 210 bp α6 (α6), and 489 bp 18S fragments (18S) is indicated along with that of the most relevant markers (on the left). (C) Transfection of both -954α5-CAT and -92α5-CAT into T97, T98, T108, and T115 cells. *CAT activities [expressed as (%CAT/4 h/100 μg proteins)/ng hGH] statistically different from those measured in T98 cells. (D) Transfection of -92α5-CAT, -132α5-CAT, and -954α5-CAT into T97, T98, T108, and T115 cell lines. CAT activities statistically different from those measured with the -92α5-CAT (*) or the -132α5-CAT (†) construct ($P < 0.05$; paired samples, *t* test) are indicated. (E) Transfection of -132α5CAT, or its derivatives bearing mutations into either the proximal (-132α5Mp) or the distal (-132α5Md) Sp1 site, or mutated into both Sp1 sites (-132α5Mp+d) in T97 and T108 cells. *CAT activities statistically different from those measured with -132α5-CAT.

individually did not significantly alter the activity directed by the -92α5-CAT construct whereas mutations that prevent binding of Sp1 to both sites reduced CAT activity by three- to fivefold (Figure 2E), indicating that both sites function in a redundant manner.

Expression and DNA-binding properties of Sp1, Sp3, AP-1, and NFI in uveal melanoma cell lines

Basal transcription of the α5 gene does not solely rely on the only action of Sp1 as binding of the TFs Sp3, NFI, and AP-1 to a short regulatory element from the α5 promoter located between positions −40 and −75 has also been demonstrated (Gingras et al., 2003, 2009; Larouche et al., 2000). We therefore monitored both DNA binding and expression of these TFs, including Sp1, by electrophoretic mobility shift assays (EMSA) using nuclear extracts prepared from T97, T98, T108, and T115 cells. Incubation of the Sp1-labeled probe with nuclear proteins from these cell lines revealed the formation of the typical Sp1 and Sp3 complexes in EMSA (Figure 3A, left). However, DNA binding of Sp3 was much stronger in T108 and T115 cells (lanes 4 and 5) than T97 and T98 cells (lanes 2 and 3). Formation of these complexes was found to be specific as only the unlabeled Sp1 (lane 6) but not the AP-1 (lane 7) oligonucleotide competed for the formation of the Sp1/Sp3-shifted bands. The identity of the TFs yielding these complexes was demonstrated through supershift analyses. Indeed, addition of antibodies (Ab) against either Sp1 or Sp3 reduced the formation of the corresponding complexes in EMSA and yielded new supershifted complexes with slower electrophoretic mobilities in both T97 and T108 cells. The use of an antibody against the unrelated TF NFI did not alter the formation of both complexes and yielded no supershifted signal (lanes 10 and 13). Western blot analyses confirmed that both T108 and T115 cells express levels of Sp3 higher than in T97 and T98 cells (Figure 3A, right). Whereas Sp1 appeared as a single band on the blot, Sp3 yielded two distinct doublets of proteins near 110 and 80 kDa. These additional protein bands have been previously reported to result from two additional internal methionine codons that are very efficiently used by the cell to yield Sp3 derivative proteins with molecular masses of 78 and 80 kDa (Kennett et al., 1997). Because of their very near molecular masses, both proteins appeared as

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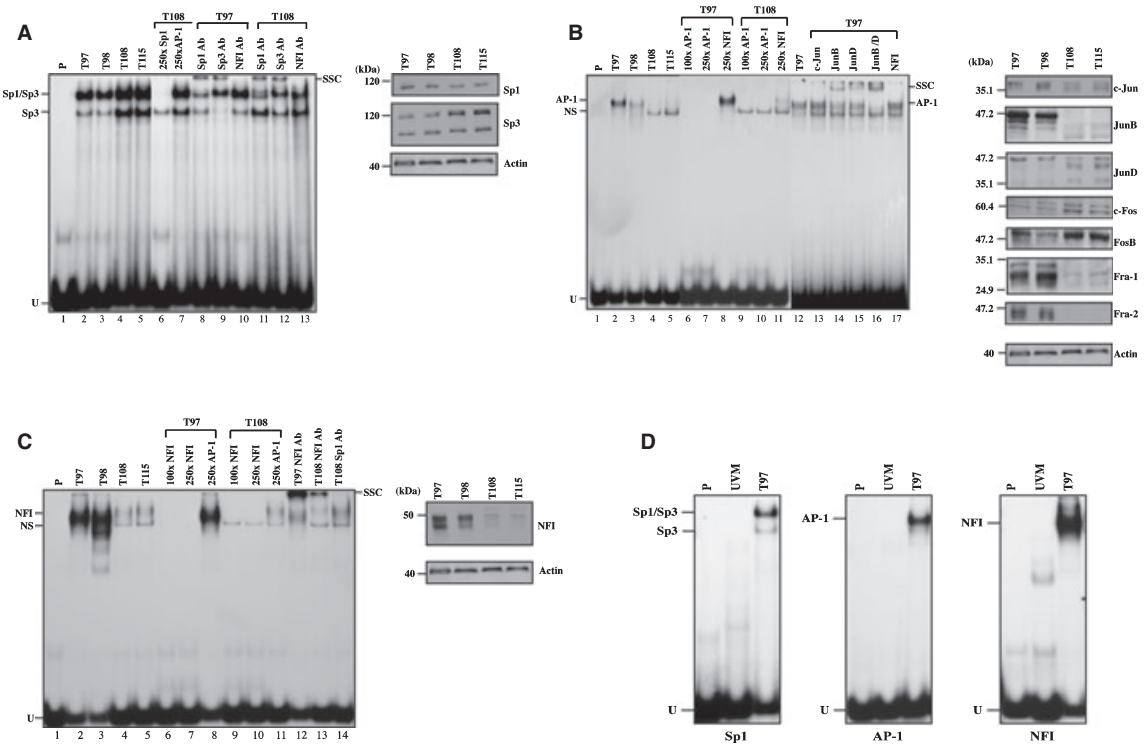

Figure 3. EMSA and Western blot analyses of Sp1/Sp3, AP-1 and NFI. Nuclear proteins (10 μg) from T97, T98, T108, and T115 cells were incubated with labeled probes bearing the target sites for Sp1/Sp3 (panel A, left), AP-1 (panel B, left), or NFI (panel C, left), either alone, or with unlabeled competitors (Sp1, AP-1, NFI; 100- or 250-fold molar excesses). When indicated, antibodies against Sp1, Sp3, and NFI or against the AP-1 subunits c-Jun, JunB, or JunD were added prior to separation of the complexes by EMSA. SSC, supershifted complexes; U, free probe; NS, non-specific complexes; P, labeled probe alone. Western blot conducted on 10 μg nuclear extracts from T97, T98, T108, and T115 cells using antibodies against Sp1, Sp3 (panel A, right), c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, Fra-2 (panel B, right), and NFI (panel C, right). (D) Comparative analysis of Sp1/Sp3, AP-1, and NFI binding using nuclear extracts from uveal melanocytes and T97 cells. AP-1, activator protein 1; EMSA, electrophoretic mobility shift assays; NFI, nuclear factor I.

a single band on the blot on Figure 3A (right). The doublet of bands (near 110 kDa) appearing at the top of the blot is also very frequently observed and is believed to correspond to glycosylated versus non-glycosylated full-length Sp3 protein.

Interestingly, incubation of the nuclear extracts with the AP-1-labeled probe revealed the formation of an AP-1 complex with a strong and moderate intensity in the T97 and T98 extracts, respectively, but yielded only a faint signal with the T108 and T115 extracts (Figure 3B, left; lanes 2–5). Formation of this complex was found to be specific as only the unlabeled AP-1 oligonucleotide (lanes 6, 7, 9, and 10) but not that bearing the NFI site (lanes 8 and 11) could compete for the formation of the AP-1 complex. Furthermore, antibodies directed against JunB or JunD proved efficient at supershifting the AP-1 complex in EMSA (lanes 14 and 15). Addition of both the JunB and JunD antibodies together (lane 16) almost entirely prevented the formation of the AP-1 complex, whereas neither the c-Jun (lane 13) nor the NFI (lane 17) antibodies had any influence on its formation. Western blot analyses of the AP-1-constituting subunits indicated that T97 and T98 cells express all Jun and Fos subunits to similar levels, although both JunB and Fra-2 yielded the strongest signals (Figure 3B, right). Similarly, T108 and T115 cells also express all Jun subunits, although both c-Jun and JunD predominate over JunB. However, unlike T97 and T98 cells, both the T108 and T115 cells had a low level of Fra-1 and no Fra-2 expression, whereas both c-Fos and FosB appear to be the predominating Fos subunits expressed by these cell lines.

Of all these TFs, the most striking difference was observed with NFI. Indeed, a strong NFI binding was observed with the extracts from T97 (Figure 3C, left; lane 2) and T98 (lane 3) cells whereas those from T108 (lane 4) and T115 cells (lane 5) only yielded a weak signal, a result also confirmed by Western blot analyses (Figure 3C, right). Again, formation of these complexes was found to be specific as only the unlabeled NFI oligonucleotide (T97: lanes 5 and 6; T108: lanes 10 and 11) but not that bearing the AP-1 site (lanes 8 and 11) competed for the formation of the NFI-shifted bands. Furthermore, addition of an antibody directed against all NFI isoforms efficiently supershifted the NFI/probe complex with both T97 (lane 12) and T108

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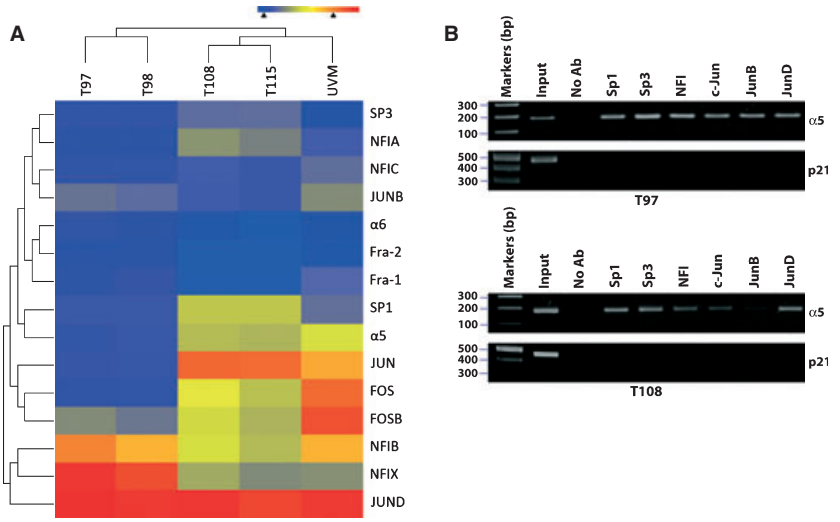

Figure 4. Microarray and ChIP analyses of transcription factors binding to the α5 promoter. (A) Heat map representation of the transcriptional profiles of T97, T98, T108, T115, and uveal melanocytes for 15 selected genes of interest. Hierarchical clustering of the cell lines and genes are shown above and on the left of the heat map, respectively. (B) Chromatin immunoprecipitation of Sp1, Sp3, NFI, JunB, c-Jun, JunD, FosB, c-fos, Fra-1, and Fra-2 in T97 and T108 cells. Input: total chromatin without immunoprecipitation (positive control); No Ab: immunoprecipitation without antibody (negative control). PCR of the p21 upstream region is used as a negative control for all antibodies. NFI, nuclear factor I.

(lane 13) nuclear extracts whereas the Sp1 Ab could not (lane 14).

Nuclear extracts from UVM yielded no DNA/protein complexes similar to those observed for Sp1, Sp3, NFI, or AP-1 (Figure 3D). However, weak, faster-migrating DNA/protein complexes likely corresponding to TF degradation products can be observed for both Sp1 and NFI.

To further assess the variations at the transcriptional level of these TFs between the highly (T97 and T98) and poorly (T108 and T115) tumorigenic cell lines, gene expression profiling by microarray analyses was next conducted on total RNA prepared from each cell line. Clustering of the in vivo microarray data for these particular TFs between all cell lines into a single heat map (Figure 4A) corroborated their expression levels. The expression pattern for these TFs genes turned out to be similar in pairs (T97/T98 and T108/T115 cell lines), but different between the two pairs and somewhat variable when compared with the UVM. Both T108 and T115 cells express levels of Sp1 and Sp3 higher than in T97 and T98 cells. Interestingly, a marked increase in the expression of both NFIB and NFIX, which turned out to be the predominating NFI isoforms transcribed, is observed in T97 and T98 cells. Gene profiling analyses also suggest that the predominating AP-1 species expressed by T97 and T98 cells are likely composed of either JunD or JunB associated with FosB as the expression of their corresponding transcripts predominates in these cells. On the other hand, transcription of the Jun subunits JunD and c-Jun and the fos subunits c-Fos and FosB clearly predominates in both T108 and T115 cells, suggesting that the AP-1 proteins expressed by these cells is likely made up of a combination of these subunits. With the only exception of Sp3, analysis of these TFs mRNA transcripts by qPCR entirely supported these microarray data (Table 1). Gene profiling also confirmed the expression of higher levels of the α5 transcript in T108 and T115 cells whereas all cell lines expressed fairly stable, low levels of the α6 transcript (Figure 4A).

Transcription factors occupancy of the α5 promoter was next examined in vivo in T97 and T108 cells by ChIP. Antibodies against Sp1, Sp3, NFI and the AP-1 subunits c-Jun, JunB, and JunD all enriched the α5 promoter sequence in T97 cells, indicating that this genomic area is bound in vivo by these TFs (Figure 4B). In contrast, none of these Abs could enrich a known negative control region (Ouellet et al., 2006) located ~2 Kbp upstream from the p21 gene promoter. Interestingly, binding of NFI to the α5 promoter was markedly reduced in T108 cells relative to the level observed in T97 cells upon normalization to the input DNA (30-fold decrease as determined by densitometric analysis–data not shown) (Figure 4B). Besides, only the JunD Ab, and to a lesser extent also the c-Jun Ab, efficiently enriched the α5 promoter sequence in T108 cells, therefore suggesting that JunD is the predominant constituting Jun subunit of the AP-1 heterodimer that binds the α5 promoter. Again, none of these Abs could enrich the upstream region from the p21 gene, thereby demonstrating the specificity of the ChIP.

Mutational analysis of the Sp1, NFI, and AP-1 sites from the α5 promoter

As uveal melanoma cell lines with distinct tumorigenic properties also express various levels of Sp1, NFI, and AP-1, we next assessed the regulatory influence each of these TFs exert on α5 gene transcription by transfecting both T97 and T108 cells with derivatives of the -92α5-CAT construct that bear mutations into each of these TF-binding sites. Mutating the NFI site (in -92α5/NFIm) caused two- and threefold increases in α5 promoter activity in both T97 and T108 cells, respectively, suggesting that NFI is a potent repressor of α5 transcription in both cell lines (Figure 5A). Mutating the

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Table 1. qPCR analysis of Sp1, Sp3, NFI, AP-1 and α5 expression in uveal melanoma cell lines

	Sp1	Sp3	NFI-A	NFI-B	NFI-C	NFI-X	c-Jun	JunB	JunD	c-Fos	FosB	Fra1	Fra2	α5
Cell line														
T97	N.D.	1.6 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	N.D.	0.8 ± 0.1	N.D.	1.2 ± 0.3	1.1 ± 0.1	N.D.	N.D.	N.D.	N.D.	0.4 ± 0.1
T108	0.4 ± 0.1	0.4 ± 0.2	2.0 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.8 ± 1.1	0.1 ± 1.4	0.1 ± 0.1	N.D.	1.7 ± 0.1
Ratio (T97/T108)	T108 > T97	4.0	0.5	2.6	T108 > T97	3.5	T108 > T97	5.8	7.7	T108 > T97	T108 > T97	T108 > T97	N.D.	4.8

Each value shown is expressed as the ratio of the quantity mean for each transcription factor normalized to the 18S internal control. Mean standard deviation is also provided. Shadowed data indicate that the values obtained with the T97 mRNAs are not detectable (N.D.). They have been provided solely to indicate that the Sp1, NFI-C, c-Jun, c-Fos, FosB, and Fra1 transcripts are more abundant in T108 than in T97 cells.
AP-1, activator protein 1; NFI, nuclear factor I.

Sp1 site (in -92α5/Sp1m) reduced the α5 promoter activity by fivefold in T108 but entirely abolished it in T97 cells (Figure 5A). However, this entire lack of promoter activity most likely reflects an improved accessibility of NFI to its target site when Sp1 can no longer interact with the α5 basal promoter as both TFs were reported to compete with each other for the availability of their respective target site in the α5 promoter (Gingras et al., 2009). Interestingly, mutation of the AP-1 site (in -92α5/AP-1m) reduced the promoter activity by fourfold in T97 cells but only had a weak influence in T108 cells (Figure 5A), which is consistent with the variations observed in the DNA-binding properties of AP-1 between T97 and T108 cells (Figure 3A). Mutating both the NFI and AP-1 sites, preserving only the Sp1 site, increased CAT activity in T97 and T108 cells by 3- and 16-fold, respectively (Figure 5B). Similarly, preserving only the AP-1 site resulted in a 3- and 22-fold increase in T97 and T108 cells, respectively. Interestingly, preserving only the NFI site entirely abolished α5 promoter function in T97 cells but only reduced it by twofold in T108 cells, a result consistent with the variations observed in NFI protein levels and DNA binding between T97 and T108 cells (Figure 3C).

Restoring expression of α5 in T97 cells alters its tumorigenic properties in vivo

We next investigated in more detail whether the suppression of α5 expression is directly related to the tumorigenic properties of T97 cells. We therefore stably transfected a recombinant construct bearing the entire human α5 cDNA fused to the CMV promoter that encodes high levels of α5 expression in T97 cells and injected the T97α5$^+$ derivatives, along with parental T97 cells that have stably integrated the empty vector (T97α5vect), in immunodeficient mice. Two distinct mixed α5-positive, T97 subpopulations [designated T97α5$^+$(1) and T97α5$^+$(2)] were enriched by this procedure. Both T97α5$^+$(1) and T97α5$^+$(2) cell lines are morphologically very different from the parental T97 cells in that they are more elongated and far less epithelioid than T97 cells are (Figure 6A). Moreover, restoring α5 expression in these cells also dramatically increased their cell doubling time from 17 h in both the parental T97 and T97vect cell lines to 193 and 99 h in T97α5$^+$(1) and T97α5$^+$(2) cells, respectively. Detection of high levels of the α5 mRNA transcript into T97α5$^+$(1) and T97α5$^+$(2) but not with T97 and T97vect cells was confirmed by both semi-quantitative RT-PCR (Figure 6B) and gene profiling by microarrays (Figure 6C). Moreover, qPCR analyses indicated that T97α5$^+$(1) and T97α5$^+$(2) cells respectively, express 448 and 10 times more α5 mRNA transcript than parental T97 cells. Western blot analyses also confirmed the rescue of the α5 integrin subunit expression at the protein level in T97α5$^+$(1) and T97α5$^+$(2) cells whereas both T97 and T97vect cells stained negative for α5 (Figure 6D). Most of all, neither

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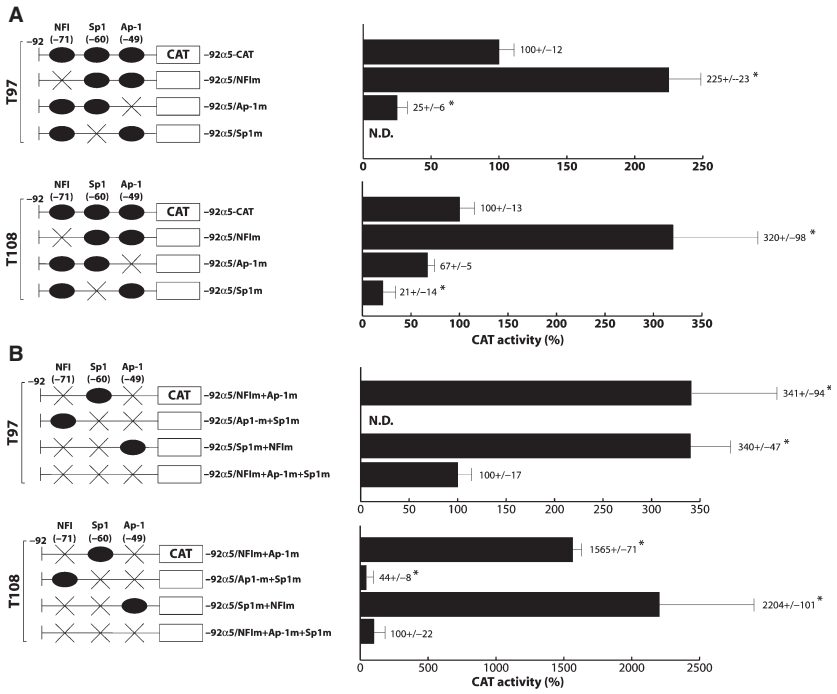

Figure 5. Mutational analyses of the Sp1, AP-1, and NFI sites from the α5 promoter. The -92α5-CAT construct or derivatives bearing mutations (X) in the AP-1, Sp1, and NFI sites either individually (A) or in combination (B) were transfected into T97 and T108 cells. CAT activities were determined and expressed relative to either wild-type -92α5 (in panel A) or to the triple-mutant 92α5/Sp1m+AP-1m+NFIm (in panel B). *CAT activities statistically different from those measured with either -92α5 (A) or -92α5/Sp1m+AP-1m+NFIm (B). AP-1, activator protein 1; NFI, nuclear factor I.

T97α5$^+$(1) nor T97α5$^+$(2) cells could yield any detectable tumors when injected s.c. into athymic nude mice, whereas α5-negative T97vect cells produced subcutaneous tumors in both flanks of all injected animals (average tumor volume of 427 mm^3 at 38 days following injections) (Figure 6E).

Discussion

In the present study, we characterized four new primary tumor-derived, uveal melanoma cell lines with very distinctive tumorigenic properties. We demonstrated that expression of the α5 integrin subunit is inversely related to their tumorigenic properties and correlated the variations in α5 gene expression with alterations in the expression of the TFs (Sp1, Sp3, AP-1, and NFI) that participate in the transcriptional regulation of that gene.

All four cell lines (T97, T98, T108, and T115) exhibited characteristics typical of cancer cells, such as a mixed phenotype that includes epithelioid cells, the ability to grow as colonies in soft agar, and a short doubling time. Furthermore, all cell lines expressed protein markers that are typically expressed by human melanomas. From these four cell lines, only the T97 and T98 cells could produce large tumors in vivo whereas immunodeficient mice injected with T108 and T115 cells developed only a few, small, or barely detectable tumors. Interestingly, the formation of vascular mimicry patterns could be reproduced in the tumors from the mice injected with T97 cells. Whereas many studies suggested that cancer cells produce and organize the ECM typical of such structures (Folberg et al., 2000; Maniotis et al., 1999, 2002), others claim exactly the opposite and suggest these patterns are fibrovascular septa that arise from a host response to the tumor (Ruiter et al., 2002). In support of the former hypothesis, Folberg et al. (Folberg et al., 2007) recently demonstrated that the liver vasculogenic mimicry patterns produced by injection of highly invasive human uveal melanoma cell lines into athymic CB17 SCID mice contained laminin of human and not mouse origin. Although the composition of the looping mimicry patterns was found to be particularly rich in laminin, type I collagen, and FN (Lin et al., 2005), yet its predominant constituent apparently turned out to be FN (Lin et al., 2005). Consequently, the formation of these FN-rich vasculogenic structures might be intimately related to the pattern of FN integrin receptors present at the cell surface of uveal melanoma cells. Although nine distinct integrins can recognize FN with varying affinities, yet the major FN-binding integrin is unquestionably α5β1 (reviewed in Vigneault et al., 2007). The data presented in this study indicate clearly that the highly tumorigenic cell lines T97 and T98 express very low levels of this integrin while, on the contrary, this receptor is highly expressed in the low tumorigenic T108 and T115 cells. Interestingly, all the patients from our study who died until now of metastatic lesions because of the dissemination of the cancer cells from the primary uveal melanoma are also those that yielded cell lines with low levels of α5 expression (SP8.0, TP17, and TP31; see Table S1). The existence of a reverse relationship between α5β1 expression and cancer cell aggressiveness is not unique to uveal melanoma as it was reported for other human

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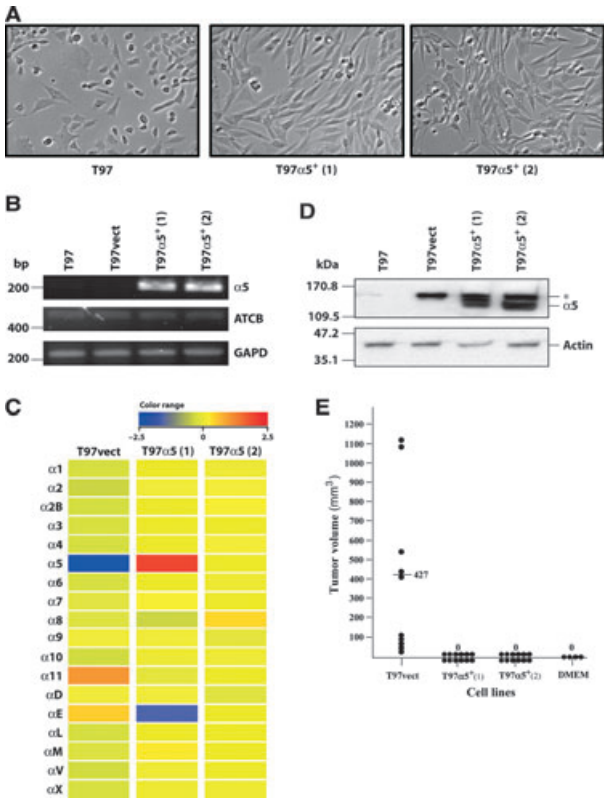

Figure 6. Influence of overexpression of α5 on the tumorigenic properties of the T97 cell line. (A) Phase contrast micrographs of monolayer cultures of the parental T97 cells and its derivative T97α5+(1) and T97α5+(2) cell lines. Magnification: 200×. (B) RT-PCR analyses of the α5 transcript in T97, T97vect, T97α5+(1), and T97α5+(2) cell lines. The position of the amplified 222bp α5, 449 bp actin (ATCB), and 220 bp GAPD fragments is indicated along with that of the most relevant markers (on the left). (C) Heat map representation of the union of every fivefold or more variation in the expression of all the human α integrin subunit genes for both T97α5+(1) and T97α5+(2) cell lines against T97 parental cells. The position of the α5 integrin subunit is indicated along with that of an unrelated protein (*) encoded by the empty vector and recognized by the P1D6 antibody directed against the α5 subunit. (D) Western blot conducted on 10-μg total protein extracts from T97vect, T97α5+(1), and T97α5+(2) cells using antibodies against α5 and actin. (E) Histogram that gives the tumor volume (mm^3) for each of the nude mice injected subcutaneously with the T97, T97vect, T97α5+(1), and T97α5+(2) cells. The horizontal bars indicate the mean tumor volumes.

and murine tumors (Stallmach et al., 1992; Weinel et al., 1992; Zutter et al., 1993). It is interesting to point out that restoration of α5 expression in the aggressive T97 cell line entirely abolished their ability to produce subcutaneous tumors in immunodeficient mice, suggesting that the lack of anchorage dependency plays a pivotal function in tumorigenic properties of these cells. In support of our results, restoration of α5β1 expression in Chinese Hamster Ovary (CHO) cells as well as colon cancer cells that frequently do not express this integrin was also reported to abrogate their anchorage-independent growth and tumorigenicity (Giancotti and Ruoslahti, 1990; Stallmach et al., 1994; Varner et al., 1995). Furthermore, osteosarcoma (Dedhar et al., 1987) and erythroleukemia cells (Symington, 1990) that express high levels of this integrin were also found to attach more firmly to FN and to be less tumorigenic. Therefore, the FN-α5β1 relationship likely influences both the tumorigenic and metastatic properties of uveal melanoma as well.

While expression and DNA binding of Sp1 and Sp3 are only moderately altered in T97/T98 relative to T108/T115 cells, the scenario is far more complex for AP-1 and NFI as dramatic alterations are observed between highly (T97/T98) and poorly (T108/T115) tumorigenic cell lines. Indeed, the most striking change is observed for NFI as both its DNA binding and expression, which are particularly elevated in the aggressive cell lines T97 and T98, are almost entirely lost in non-aggressive T108 and T115 cells. Data from gene profiling by microarray and qPCR analyses suggested that this might be the consequence of a dramatic change in the expression of the NFI-B and NFI-X isoforms. Knocking downexpression of NFI in vivo through lentivirus-mediated overexpression of shRNAs would certainly prove to be highly informative to clearly establish a causal relationship between NFI expression and repression of the α5 gene. However, as all four NFI isoforms are expressed to different levels by all uveal melanoma cell lines used in this study, one would need to simultaneously suppress the expression of the four NFI genes to clearly evaluate the contribution of this TF on α5 gene transcription. Deciphering the contribution of AP-1 to basal α5 promoter activity is by far a much more challenging task primarily because of its complex heterodimeric composition. Regardless that the DNA-binding properties of AP-1 are strongly abrogated in T108 and T115 cells, yet most of its constituting subunits are obviously expressed in these cells. Although AP-1 most likely plays a major role in α5 gene transcription, the exact nature of the Jun and Fos subunits that constitute the AP-1 heterodimer in the various cell types examined yet remains elusive. Most likely, the predominating AP-1 species expressed by T97 cells is composed of the JunD/FosB or JunB/FosB heterodimers, whereas the AP-1 protein is made up of JunD/FosB in T108 cells.

Based on the results presented here, we believe these human uveal melanoma cell lines shall prove particularly useful as cellular models to investigate the function played by the α5β1 integrin and the TFs (for instance, Sp1, Sp3, AP-1, and NFI) that regulate the expression of its α5 subunit, in the transformation of the non-neoplasic UVM into invasive uveal melanoma. As most of these TFs also regulate the expression of target genes encoding products other than integrins, a thorough analysis by gene profiling on a more exhaustive array of uveal melanoma and cancer cell lines will

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likely help in identifying potentially interesting markers for the early diagnosis of this type of cancer.

Methods

Cell lines

The T97, T98, T108, and T115 cell lines were cultured from the primary tumor of four individual donors diagnosed with uveal melanoma as described previously (Beliveau et al., 2000) (Table S1). All uveal melanoma cell lines were maintained in DMEM/F12 medium (Invitrogen Canada Inc, Burlington, ON, Canada), whereas human Jurkat cells (ATCC TIB 152) were grown in RPMI-1640 (Invitrogen Canada Inc). Both culture media were supplemented with 10% FBS (Gemini; NorthBio, Toronto, ON, Canada), and cells were grown at 37°C under 5% CO_2. Isolation and culture of normal UVM were performed according to the procedure described by Hu et al. (1993) from a total of 27 post-mortem eyes from human donors aged 9–73 yr (median of 42 yr old) and obtained from the Banque d'Yeux Nationale de Québec (QC, Canada). For each individual experiment, cells were pooled to avoid any donor-specific variations that may occur between patients of different ages and health status. Analyses in flow cytometry were conducted on three different batches of UVM each cultured from the pool of two donors (median age: 51 yr old); RT-PCR was conducted on a pool of cells from 18 donors (median age: 31 yr old); preparation of crude nuclear extracts was conducted on two different batches of pooled cells: batch 1 was prepared from four donors (median age: 40 yr old) and batch 2 from three donors (median age: 47 yr old).

T97 cells stably expressing the human α5 subunit were produced as detailed previously (Beliveau et al., 2001) by using the Tet-off vector system kindly provided by Dr M. Brattain (Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, OH, USA). Transfected cells received 20 μg of the linearized α5 expression plasmid and 2 μg of the linearized blasticidin-encoding plasmid (plentiV5/GFPtag; a kind gift of Dr. François Boudreault, département d'Anatomie-Biologie Cellulaire, Sherbrooke University, Sherbrooke, QC, Canada). Blasticidin (4 μg/ml) was added to the culture medium 24 h following transfection and maintained for 2 weeks. α5-positive/blasticidin-resistant T97 cells (designated as T97α5$^+$) were then sorted out by flow cytometry (Epics XL, Coulter Electronic) and further expanded prior to their s.c. injection into nude mice. Two different cell lines that express different levels of the α5 subunit were produced using this approach and were designated T97α5$^+$(1) and T97α5$^+$(2).

Flow cytometry

Expression of the α5 and α6 integrin subunits was monitored by flow cytometry in T97, T98, T108, and T115 cells as described (Beliveau et al., 2000) using monoclonal antibodies directed against the human integrin subunits α5 and α6 (P1D6 and NKI-GoH3, respectively; Chemicon, Temecula, CA, USA) (also refer to supporting methods S1).

Plasmids, oligonucleotides, cell transfections and CAT assays

The plasmids -954α5-CAT, -132α5-CAT, and -92α5-CAT have been previously described (Birkenmeier et al., 1991; Gaudreault et al., 2007). The oligonucleotides bearing the DNA-binding sites for Sp1 (Dynan and Tjian, 1983), NFI (De Vries et al., 1987), and AP-1 (Corbi et al., 2000; Gingras et al., 2009) were chemically synthesized using a Biosearch 8700 apparatus (Millipore, distributed by Fisher, Ottawa, ON, Canada). All recombinant plasmids were transfected using Lipofectamine (Gibco BRL, Invitrogen, Burlington, ON, Canada) in uveal melanoma cell lines grown to 80% confluence. CAT activities were determined and normalized to secreted human growth hormone (hGH) as described (Pothier et al., 1992). Each CAT value corresponds to the mean of at least three separate transfections performed in triplicate. Student's t test was performed for comparison of the groups. Differences were considered to be statistically significant at $P < 0.05$. All data are expressed as mean ± SD.

Tumorigenicity assays and immunohistochemical analyses

Approximately 7×10^5 cells (T97, T98, T108, and T115) in complete DMEM were injected subcutaneously into the flanks of Crl:CD1®-nuBR athymic nude mice (Charles River, St-Constant, QC, Canada). Eight 7-week-old female mice were used for each cell line. For the experiment conducted with the T97α5$^+$ cell line, 1×10^6 cells were injected into both flanks of six athymic nude mice. Both T97 cells stably transfected solely with the empty Tet-off vector (T97vect) and wild-type T97 cells were also injected as controls. Thirty days following injection, mice were transcardially perfused (Villeneuve et al., 2008) before they were sacrificed and the tumors excised. Tumors were embedded in paraffin for their immunohistochemical analysis on 5-μM-thick sections. All sections were first stained with hematoxilin/eosin for histological analysis. The slides were deparaffinized and treated with 3% H_2O_2. Heat-induced epitope retrieval was performed with citrate buffer at 95.5°C. Immunohistochemical analyses were then performed using a diaminobenzidine biotin/streptavidine complex with rabbit antibodies raised against a glycoprotein from the premelanosome (HMB45 Ab) or the S100 protein (M-0634 and Z0311, respectively; Dako Canada, Inc., Mississauga, ON, Canada) or a mouse monoclonal antibody against the MART-1 protein (A103; Invitrogen Canada Inc) as primary antibodies.

Western blot

Western blots were conducted as described (Larouche et al., 2000) using 30-μg nuclear proteins with the following primary antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA, except for CLT-9001 which came from Jackson Immuno Research Laboratories): rabbit polyclonal antibodies against Sp1 (sc-59, 1:5000), Sp3 (sc-644, 1:4000), NFI (sc-5567, 1:1200), c-jun (sc-45, 1:3000), JunD (sc-74, 1:2000), FosB (sc-7203, 1:900), c-Fos (sc-52, 1:300), Fra-1 (sc-605, 1:1000), or Fra-2 (sc-604, 1:1200), or mouse monoclonal antibodies against JunB (sc-46 1:3000) and actin (CLT 9001; 1:35000) as well as a peroxidase-conjugated AffiniPure Goat secondary antibody against either mouse or rabbit IgG (1:1000 dilution). The labelling was revealed using a Detection kit (Amersham, distributed by GE Healthcare, Baie d'Urfé, Canada) as described (Gaudreault et al., 2007; Gingras et al., 2003). For detection of the α5 integrin subunit in T97α5$^+$(1) and T97α5$^+$(2) cells, a monoclonal antibody (final concentration of 10 μg/ml) directed against the human integrin subunits α5 (P1D6; Chemicon) was used as the primary antibody.

Nuclear extracts and electrophoretic mobility shift assays

Nuclear extracts were prepared from UVM and uveal melanoma cell lines as described (Gaudreault et al., 2007; Gingras et al., 2003). The oligonucleotides bearing the Sp1-, NFI-, and AP-1-binding sites were 5′ ^{32}P-end-labeled and used as probes in EMSA (Gaudreault et al., 2007; Gingras et al., 2003). Supershift experiments in electrophoretic mobility shift assays were conducted by incubating 5 μg nuclear proteins in the presence of either no or polyclonal antibodies against the TFs Sp1, Sp3, NFI, c-Jun, JunB, and JunD.

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RT-PCR and chromatin immunoprecipitation (ChIP) assays

RT-PCR was performed as described (Proulx et al., 2004) using the QuantumRNA 18S Internal standards protocol with the primer sets shown on Table S2. The ChIP analyses were conducted on both T97 and T108 cells with antibodies against the different TFs as previously reported (Gaudreault et al., 2007; Ouellet et al., 2006). The resulting DNA was analyzed by PCR using primers (ITGA5-U: 5′CTTAGGGGTGGGGGACGC-3′ and ITGA5-L: 5′CGCCCGCTCTTCCCTGTC-3′) spanning the α5 gene promoter.

Quantitative PCR

Quantitative PCR (qPCR) analyses were carried out following a previously described procedure (Woelk et al., 2004). Briefly, total RNA was isolated from both T97 and T108 cell lines using the RNA Stabilization Reagent and Rneasy kit (cat. # 74104; Qiagen, Toronto, ON, Canada). RNA quantity and quality were assessed using an Agilent Technologies 2100 bioanalyzer and RNA 6000 Nano LabChip kit (Agilent, Mountain View, CA, USA). Reverse transcription was performed using random hexamer primers following the manufacturer's protocol for synthesis of the first-strand cDNA (Superscript II; Invitrogen Canada Inc). Equal amounts of cDNA were run in triplicate and amplified in a 15 μl reaction containing 7.5 μl of 2× Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 10 nM of Z-tailed forward primer, 100 nM of reverse primer, 100 nM of Amplifluor Uniprimer probe (Chemicon), and 2 μl of DNA target. Moreover, no-template controls were used as recommended. The mixture was incubated at 50°C for 2 min, at 95°C for 4 min, and then cycled at 95°C for 15 s and at 55°C for 40 s 55 times using the Applied Biosystems Prism 7900 Sequence Detector. Amplification efficiencies were validated and normalized to ribosomal 18S, and quantities of the target genes were calculated according to a standard curve. Primers were designed using Primer Express 2.0 (Applied Biosystems). Amplicons were detected using the Amplifuor UniPrimer system, and forward primers used contained a Z sequence: 5-ACTGAACCTGACCGTACA-3 (Nuovo et al., 1999). The specific primers used are listed on Table S2.

Gene expression profiling

Total RNA was isolated using the mirVana miRNA Isolation kit (Ambion, Austin, TX, USA). Biotinylated (Illumina protocol) or cyanine 3-CTP (Agilent protocol)-labeled cRNA targets were prepared from 150 ng (Illumina protocol) or 25 ng (Agilent protocol) of total RNA, using either the Illumina TotalPrep RNA Amplification kit (Ambion) or the Agilent One-Color Microarray-Based Gene Expression Analysis kit (Agilent technologies Canada, Inc., Mississauga, ON, Canada), respectively. Then either 1.5 μg or 600 ng cRNA was incubated on a HumanHT-12 v3 Expression BeadChip arrays (48 804 probes; Illumina, San Diego, CA, USA) or a G4851A SurePrint G3 Human GE 8x60K array slide (60 000 probes, Agilent technologies), respectively. Slides were then hybridized (Agilent protocol), washed, stained (Illumina protocol), and scanned on either an Illumina BeadStation 500 or an Agilent C Scanner according to the manufacturer's instructions. Data were finally analyzed using either the ArrayStar V3.0 (DNASTAR, Madison, WI, USA) or the GeneSpring GXI (Agilent Technologies) software for scatter plot and generation of the heat maps of selected genes of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplementary methods.

Figure S1. Expression of melanoma-specific markers and growth properties of uveal melanoma cell lines.

Table S1. Summary of cases from which cell lines were derived.

Table S2. Sequences of the forward and reverse primers used for RT-PCR and q-PCR.

Table S3. Flow cytometric analysis of α5 and α6 integrin subunits expression in uveal melanoma cell lines.

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