

Loss of AP-2 results in downregulation of c-KIT and enhancement of melanoma tumorigenicity and metastasis

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Expression of the tyrosine kinase receptor, c-KIT, progressively decreases during local tumor growth and invasion of human melanomas. We have previously shown that enforced c-KIT expression in highly metastatic cells inhibited tumor growth and metastasis in nude mice. Furthermore, the ligand for c-KIT, SCF, induces apoptosis in human melanoma cells expressing c-KIT under both *in vitro* and *in vivo* conditions. Here we show that loss of c-KIT expression in highly metastatic cells correlates with loss of expression of the transcription factor AP-2. The c-KIT promoter contains three binding sites for AP-2 and EMSA gels demonstrated that AP-2 protein binds directly to the c-KIT promoter. Transfection of wild-type AP-2 into c-KIT-negative A375SM melanoma cells activated a c-KIT promoter-driven luciferase reporter gene, while expression of a dominant-negative AP-2B in c-KIT-positive Mel-501 cells inhibited its activation. Endogenous c-KIT mRNA and expression of proteins were upregulated in AP-2-transfected cells, but not in control cells. In addition, re-expression of AP-2 in A375SM cells suppressed their tumorigenicity and metastatic potential in nude mice. These results indicate that the expression of c-KIT is highly regulated by AP-2 and that enforced AP-2 expression suppresses tumorigenicity and metastatic potential of human melanoma cells, possibly through c-KIT transactivation and SCF-induced apoptosis. Therefore, loss of AP-2 expression might be a crucial event in the development of malignant melanoma.

Keywords: apoptosis/malignant melanoma/metastasis/transcription factor/tyrosine kinase

Introduction

The molecular basis of human malignant melanoma progression has remained largely unknown despite the fact that the worldwide incidence of melanoma is increasing more than any other neoplastic disease (Kopf *et al.*, 1995). The molecular changes associated with the transition of melanoma cells from radial growth phase (RGP) to vertical growth phase (VGP, metastatic phenotype) are not well defined. Since the production of metastases depends on the completion of a multistep process involving the survival and growth of a unique subpopulation of cells

with metastatic properties (Fidler, 1990), more information is clearly needed regarding genetic changes underlying melanoma tumorigenesis and progression to provide insights into the development of this cancer.

There has, however, been some progress. Of particular relevance to this paper are recent results with the c-KIT receptor in melanoma. Expression of the tyrosine kinase receptor encoded by the *c-KIT* proto-oncogene progressively decreases during local tumor growth and invasion of human melanomas (Lassam and Bickford, 1992; Natali *et al.*, 1992; Zakut *et al.*, 1993). The proto-oncogene *c-KIT* encodes a transmembrane tyrosine-protein kinase receptor related to the PDGF/CSF-1 (c-fms) receptor subfamily (Yarden *et al.*, 1987). c-KIT has been found to play a pivotal role in the normal growth and differentiation of embryonic melanoblasts. In mice, c-KIT has been mapped to the dominant white spotting (*w*) locus (Chabot *et al.*, 1988; Geissler *et al.*, 1988), whose ligand is the product of the *sl* locus (Steel) (Zsebo *et al.*, 1990) which encodes the stem cell factor, SCF (also known as KIT-ligand, KL, steel factor or mast cell growth factor, MCF). Mutations in the (*w*) locus or (*sl*) locus (Nocka *et al.*, 1989, 1990; Reith *et al.*, 1990; Tan *et al.*, 1990; Brannan *et al.*, 1991; Flanagan *et al.*, 1991), or injection of neutralizing anti-KIT antibodies into pregnant mice (Nishikawa *et al.*, 1991) results in the piebald phenotype, characterized by white spotting of the fur and attributed to a local reduction in the number of cutaneous melanocytes. Mutations in the c-KIT receptor also have been identified in human piebald patients (Fleishman *et al.*, 1991; Gibel and Spritz, 1991; Fleischman, 1992), suggesting that normal function of c-KIT is required for human melanocyte development.

These observations raise the question as to whether malignant transformation of melanocytes may be associated with changes in the expression of the c-KIT receptor. Indeed, several recent studies have demonstrated that the progression of human cutaneous melanoma is associated with loss of expression of the c-KIT proto-oncogene. About 70% of metastatic lesions and human melanoma cell lines do not express detectable levels of the c-KIT receptor (Lassam and Bickford, 1992; Natali *et al.*, 1992; Zakut *et al.*, 1993). To provide direct evidence that c-KIT plays a role in metastasis of human melanoma, we transfected the *c-KIT* gene into c-KIT-negative, highly metastatic human melanoma cells and subsequently analyzed their tumorigenic and metastatic potential in nude mice (Huang *et al.*, 1996). Enforced c-KIT expression significantly inhibited tumor growth and metastasis. Exposure of c-KIT-positive melanoma cells *in vitro* and *in vivo* to SCF, the ligand for c-KIT, triggered apoptosis of these cells but not of normal melanocytes. These results suggest that loss of c-KIT receptor may allow malignant melanoma cells to escape SCF/c-KIT-mediated apoptosis, thus

contributing to tumor growth and eventually metastasis (Huang *et al.*, 1996).

The mechanism(s) for the loss of c-KIT gene expression during melanoma progression are unknown. The promoter of the c-KIT gene has been cloned and sequenced (Yamamoto *et al.*, 1993). The human c-KIT promoter region lacks a typical 'TATA box' but has a relatively high G+C content and strikingly, three putative AP-2-binding elements. These observations, coupled with our previous finding that highly metastatic human melanoma cells do not express the AP-2 transcription factor (Bar-Eli, 1997), led us to hypothesize that AP-2 may regulate c-KIT gene expression in human melanoma cells.

AP-2, a 52 kDa protein, was first purified from HeLa cells. Partial peptide sequences led to the isolation of the cDNA from a HeLa cell library (Williams *et al.*, 1988), and the gene was mapped to a region on the short arm of chromosome 6 near the HLA locus (Gaynor *et al.*, 1991; Mitchell *et al.*, 1991). The AP-2 protein binds to a consensus palindromic core recognition element with the sequence 5'-GCCNNGGC-3' (Williams *et al.*, 1988). Functional AP-2-binding sites have been identified in the enhancer regions of viral genes such as simian virus 40 (SV40) (Mitchell *et al.*, 1987), human T-cell leukemia virus type I (Nyborg and Dynan, 1990), and cellular genes such as murine major histocompatibility complex (H-2K^b), human metallothionein-IIa (huMTIIa), human proenkephalin, human keratin K14 genes, c-erbB-2, plasminogen activator inhibitor type I (PAI-1) and insulin-like growth factor binding protein-5 (Lee *et al.*, 1981; Haslinger and Karin, 1985; Hyman *et al.*, 1989; Leask *et al.*, 1991; Descheemaeker *et al.*, 1992; Bosher *et al.*, 1995; Duan and Clemmons, 1995). The DNA-binding domain is located within the C-terminal half of the 52 kDa protein and consists of two putative amphipathic α helices separated by an 82 amino acid intervening span that is both necessary and sufficient for homodimer formation (Williams and Tjian, 1991). An alternatively spliced AP-2 protein, AP-2B, that differs in its C-terminus and acts as dominant-negative to AP-2 has recently been cloned (Buettner *et al.*, 1993).

AP-2 activity is regulated through a number of different signal transduction pathways. Phorbol esters and signals that enhance c-AMP levels induce AP-2 activity independently of protein synthesis, whereas retinoic acid treatment of teratocarcinoma cell lines results in a transient induction of AP-2 mRNA levels on a transcriptional level (Luscher *et al.*, 1989; Buettner *et al.*, 1993).

AP-2 is involved in mediating programmed gene expression both during embryonic morphogenesis and adult cell differentiation. Using *in situ* hybridization, a restricted spatial and temporal expression pattern has been observed during murine embryogenesis. In particular, regulated AP-2 expression was observed in neural crest-derived cell lineages (from which melanocytes are derived) and in facial and limb bud mesenchyme (Mitchell *et al.*, 1991). Two recent reports of AP-2 null mutant mice have demonstrated that AP-2 is important for development of the cranial region and for midline fusions. The AP-2 null mice died at birth (Schorle *et al.*, 1996; Zhang *et al.*, 1996).

In this study, we provide the first evidence that (i) there is a direct correlation between AP-2 and c-KIT expression in human melanoma cells; (ii) transfection of highly

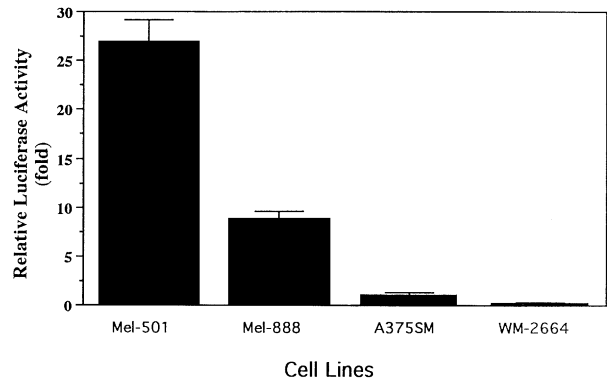


Fig. 1. Luciferase activity driven by the c-KIT promoter in human melanoma cell lines. 2 μ g of c-KIT luciferase reporter gene construct pKluc and 0.05 μ g of pB-Actin-RL (Renilla) plasmid were co-transfected into 2×10^5 melanoma cells, and incubated for 60 h at 37°C. Firefly and Renilla luciferase activities were quantified using the Dual-Luciferase Reporter Assay System. Luciferase activity was compared with c-KIT-negative A375SM cells which were given the reference value of 1. Higher luciferase activity was observed in the two c-KIT-positive cell lines, Mel-501 and Mel-888, than in the c-KIT-negative cell line, WM2664. The standard deviation bars represent replicates within the assay. This experiment is a representative of three performed.

metastatic cells (c-KIT negative, AP-2 negative) with the AP-2 gene resulted in induction of c-KIT mRNA and protein; and (iii) re-expression of AP-2 in highly metastatic melanoma cells inhibited their tumor growth and metastatic potential in nude mice, possibly through transactivation of c-KIT. Our results add weight to the hypothesis that loss of AP-2 expression is a crucial event in the development of malignant melanoma, especially since other genes involved in the progression of human melanoma such as MCAM/MUC18, E-cadherin, MMP-2 and p21/WAF-1 are also regulated by AP-2.

Results

Direct correlation between c-KIT and AP-2 expression in human melanoma cell lines

The mechanism(s) for lack of expression of c-KIT in metastatic melanoma cells is unknown. In an effort to determine the molecular basis for c-KIT's lack of expression in highly metastatic melanoma cells, we found that the c-KIT gene and its promoter in c-KIT-negative melanoma cells had no abnormalities (deletions, rearrangements or mutations) that can account for the lack of c-KIT expression (Huang *et al.*, 1996; Bar-Eli, 1997; data not shown). These observations suggest that c-KIT expression might be regulated at the transcriptional level. To test this hypothesis, we subcloned the promoter of the c-KIT gene (-1215 to +1) (Yamamoto *et al.*, 1993) in front of the luciferase reporter gene. Using the Dual-Luciferase Reporter System, we analyzed the luciferase activity driven by the c-KIT promoter in c-KIT-positive and c-KIT-negative melanoma cell lines. As shown in Figure 1, c-KIT-luciferase activity was higher in the human melanoma cell lines Mel-888 and Mel-501 (both express high levels of c-KIT mRNA) (Zakut *et al.*, 1993), as compared with the activity in c-KIT-negative A375SM cells which was given the reference value of 1. We observed low luciferase activity

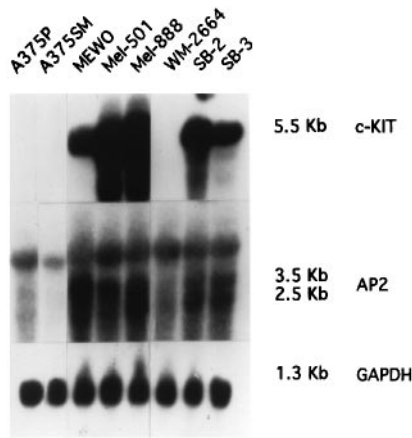


Fig. 2. Northern blot analysis for the expression of c-KIT and AP-2 in human melanoma cell lines. c-KIT-positive melanoma cell lines expressed high levels of the two AP-2 transcripts, while c-KIT-negative melanoma cell lines were either negative for AP-2 (A375SM), or expressed negligible amounts of AP-2 mRNA (A375P, WM-2664). The upper band at ~5 kb which is expressed in all lanes most likely represents the AP-2B transcript (Buettner *et al.*, 1993). The same filter was hybridized with GAPDH to verify the integrity and the amount of RNA loaded in each lane.

in the WM-2664 cell line which does not express c-KIT mRNA and protein (Gutman *et al.*, 1994). These results suggest that c-KIT expression is regulated at the transcriptional level in these melanoma cells.

The 1.2 kb c-KIT promoter lacks TATA or CCAAT boxes, is highly G+C rich, and contains binding sites for SP-1, myb, GATA-1 and three putative AP-2 sites (Yamamoto *et al.*, 1993). Deletion of the proximal 185 bp of the promoter abolished the transcription of c-KIT in HEL cells, suggesting that only the proximal 185 bp of the promoter are necessary and sufficient for c-KIT expression (Yamamoto *et al.*, 1993). As we closely examined the proximal 185 bp promoter region, two AP-2 binding sites were identified within this region with a total of three putative AP-2 binding motifs within the 1.2 kb c-KIT promoter (Figure 3C). The presence of two AP-2-binding sites within the essential region of c-KIT promoter suggested that AP-2 might regulate expression of the c-KIT gene. Indeed, during mouse development, AP-2 and c-KIT are co-expressed in several tissues including hindbrain, kidney and heart, and in neural crest-derived lineages from which melanocytes originate (Orr-Urtreger *et al.*, 1990; Mitchell *et al.*, 1991). These observations prompted us to analyze whether the progression of human melanoma is associated with changes in the expression of the AP-2 transcription factor via regulation of the c-KIT gene.

To study whether AP-2 plays a role in the regulation of c-KIT expression in human melanoma cells, we first examined the status of AP-2 in a panel of human melanoma cell lines exhibiting different metastatic capabilities in nude mice (Luca *et al.*, 1995; Bar-Eli, 1997; Xie *et al.*, 1997). Figure 2 demonstrates that expression of AP-2, correlated with the expression of c-KIT and the metastatic potential of human melanoma cells i.e. the non/low metastatic melanoma cell lines in nude mice such as MeWo, Mel-501, Mel-888, SB-2 and SB-3, expressed high levels of c-KIT and the two AP-2 transcripts. In contrast,

the cell lines derived from metastatic lesions and also metastatic in nude mice (Luca *et al.*, 1995) A375P, A375SM and WM-2664 were c-KIT negative and were also either negative for AP-2 (A375SM), or expressed negligible amounts of AP-2 mRNA (A375P, WM-2664). Expression of AP-2 in the nonmetastatic SB-2 cells and lack of expression of AP-2 in the highly metastatic A375SM cells was confirmed by Western blot analysis (Bar-Eli, 1997; see Figures 6 and 7).

Transactivation of c-KIT promoter by AP-2A and repression by the dominant-negative AP-2B gene

To assess the effect of AP-2 on c-KIT transcription, the c-KIT promoter-luciferase construct, pKLuc, was co-transfected into A375SM cells with increasing concentrations of an expression vector encoding for wild-type AP-2 (AP-2A, pSG5-AP-2) or with the control vector lacking AP-2A (pSG5). Using the β -actin-Renilla luciferase plasmid (pB-Actin-RL) vector as a control to normalize for transfection efficiency, Figure 3A shows that the luciferase activity driven by the c-KIT promoter was activated by AP-2A in A375SM cells in a dose-dependent manner. A 9.5-fold stimulation was observed in cells co-transfected with 6 μ g of the plasmid expressing the AP-2A protein, which was not detected in transfections with the parent vector. Conversely, when the pKLuc construct was co-transfected into Mel-501 cells (which express high levels of c-KIT) and increasing concentrations of AP-2B, the dominant-negative form of AP-2 (Buettner *et al.*, 1993), the luciferase activity was inhibited in these cells (Figure 3B). A 48% decrease in luciferase activity was observed with 3.5 μ g of the plasmid encoding AP-2B. No further decrease in activity was observed with higher concentrations of AP-2B. It has been shown that AP-2A and AP-2B do not interact directly. The lack of further repression of the c-KIT promoter in Mel-501 cells by AP-2B might be due to the limited amount of a putative adapter protein required for interaction between AP-2A and AP-2B (Buettner *et al.*, 1993). However, we cannot rule out the possibility that AP-2 is not the sole factor regulating c-KIT expression in these cells. These experiments indicate the presence of functional AP-2 elements within the c-KIT promoter that regulate c-KIT expression in human melanoma cell lines.

To provide direct evidence that the AP-2 sites within the 185 bp proximal c-KIT promoter actually contribute to its activity, we performed site-directed mutagenesis of the AP-2 sites located between -80 to -89 and -139 to -148 upstream of the c-KIT transcription initiation site (Figure 3C). Disruption of the upstream AP-2 site located between -139 and -148 significantly inhibited reporter activity in c-KIT-positive Mel-501 cells (Figure 3E) and in c-KIT-negative A375SM cells co-transfected with 2 μ g of expression vector for AP-2A (Figure 3D). Mutations of the downstream AP-2 binding site (-80 to -89) did not alter the luciferase activity in these cells, indicating that the AP-2 site located between -139 and -148 is crucial for the c-KIT promoter activation. This could be explained by the difference in the sequence of these two AP-2 sites in which the site located between -139 to -148 has more homology with the core AP-2 recognition sequence (Williams *et al.*, 1988).

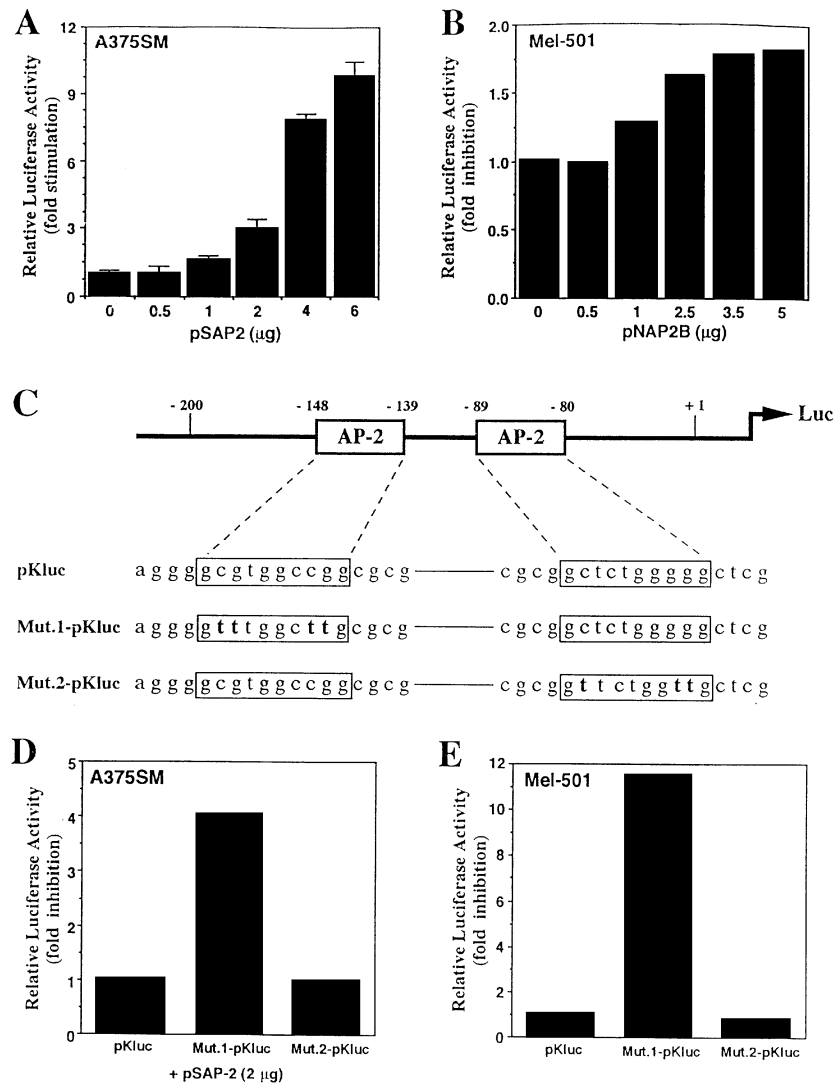


Fig 3. (A) Effect of AP-2 expression on *c-KIT* promoter. 2 µg of pKluc, 1–6 µg of pSG5-AP-2 (expression vector for wild-type AP-2) or pSG5 (naked vector) were co-transfected into A375SM cells with PB-Actin-RL to monitor transfection efficiency. Fold activation was calculated relative to naked vector transfected control cells. Luciferase activity driven by the *c-KIT* promoter was activated by wild-type AP-2 in a dose-dependent manner. The standard deviation bars represent replicates within the assay. This is one of two experiments performed. (B) Effect of dominant-negative AP-2B on *c-KIT* promoter. 2 µg of pKluc, 1–5 µg of pSG5-AP-2B (or pSG5) and 0.05 µg of pB-Actin-RL were co-transfected into Mel-501 cells. *c-KIT* promoter activity was inhibited by dominant-negative AP-2B. A 48% inhibition in luciferase activity was observed with 2.5–5 µg of AP-2B expression vectors. Fold inhibition was calculated relative to control cells transfected with the naked vector. (C) Schematic presentation of wild-type (pKluc) and mutant AP-2 DNA-binding sites –80 to –89 (Mut.1-pKluc) and –139 to –148 (Mut.2-pKluc), within the minimal region required of *c-KIT* activation. (D) Mutations within the AP-2 site located between –139 and –148 inhibited luciferase activity 4-fold in A375SM cells co-transfected with 2 µg of AP-2A expression vector. Mutations of the downstream site (–80 to –89) had no effect on the activation of the reporter gene. (E) Site-directed mutagenesis of the AP-2 site located between –139 to –148 bp upstream of the *c-KIT* initiation site significantly inhibited reporter activity (11-fold) in Mel-501 *c-KIT*-positive cells, while mutations in the AP-2 site located between –80 and –89 had no effect. Fold inhibition in (D) and (E) was calculated relative to control cells transfected with the wild-type *c-KIT* promoter pKluc.

Direct interaction of AP-2 with the *c-KIT* promoter

To determine if AP-2 transactivation of the *c-KIT* promoter was due to direct AP-2 binding to the *c-KIT* promoter, we tested a 108 bp fragment from the *c-KIT* promoter (–68 to –175, that contains two AP-2 binding sites) for reaction with recombinant human AP-2 (r-h-AP-2) on EMSA gel. Figure 4 shows that r-h-AP-2 bound directly to this fragment. This binding was abrogated by an excess of unlabeled double-stranded AP-2-binding DNA sequences, but not by the AP-1-binding DNA motif. Furthermore, the observed DNA–protein complex was super-shifted by anti-AP-2 antibody but not by anti-CREB antibody (Figure 4). In repeated EMSA gel experiments,

we were able to demonstrate that the shifted bands were eliminated by an excess of unlabeled DNA fragments corresponding to the *c-KIT* promoter region that was used as a probe (Figure 5A). To further determine the specificity of the AP-2 reaction with the *c-KIT* promoter, formation of these complexes was competed out again with an excess of unlabeled oligonucleotides corresponding to the AP-2 consensus binding motif (Figure 5B, lanes 2–4) but not with an excess of cold double-stranded oligonucleotides that were designed to harbor mutations that disrupt AP-2 binding (Figure 5B, lanes 6–8). Collectively, these data show that AP-2 bound directly to a region of the *c-KIT* promoter required for its transcription.

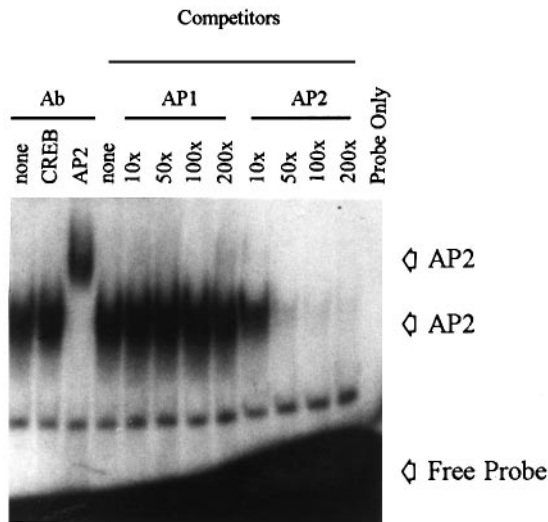


Fig. 4. Interaction of AP-2 with the *c-KIT* promoter. r-h-AP-2 binds directly to a fragment of the *c-KIT* promoter (-68 to -175) that contains two binding sites for AP-2. AP-2/*c-KIT* binding could be abrogated by an excess of AP-2 DNA binding sequences but not by AP-1 binding motifs. The specific DNA-protein complex was super-shifted by anti-AP-2 antibody but not by anti-CREB antibody.

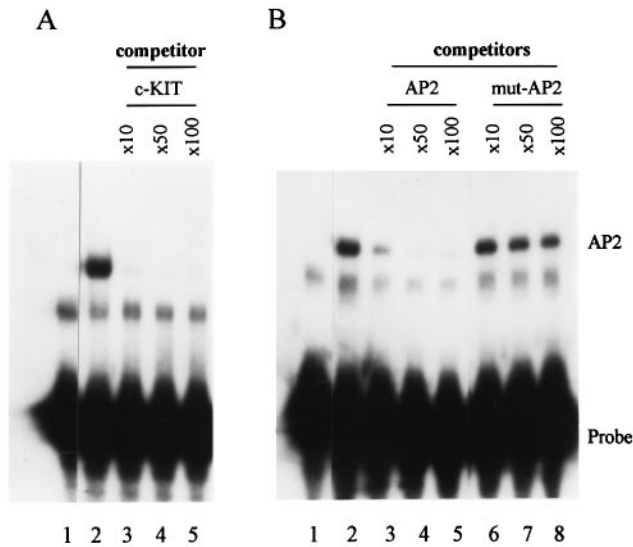


Fig. 5. Interaction of AP-2 with the *c-KIT* promoter. r-h-AP-2 bound directly to a fragment of the *c-KIT* promoter described in Figure 4. This binding could be competed out by an excess of unlabeled double-stranded DNA fragments corresponding to the *c-KIT* promoter region (A), or by oligonucleotides corresponding to the AP-2 consensus binding motif (B) (lanes 2-4), but not by an excess of oligonucleotides with mutations that disrupt AP-2 binding (B) (lanes 6-8).

Ectopic expression of AP-2 in A375SM human melanoma cells

To assess the contribution of the AP-2 transcription factor to *c-KIT* expression and to the acquisition of the metastatic phenotype in human melanoma cells, we decided to re-express AP-2 in A375SM cells. A375SM cells are highly metastatic in nude mice (Gutman *et al.*, 1994; Luca *et al.*, 1995; Xie *et al.*, 1997) and also *c-KIT* negative, and they express low or negligible levels of endogenous AP-2 (Figure 6A and B). Following gene transfection with either an expression vector carrying a full-length human AP-2A

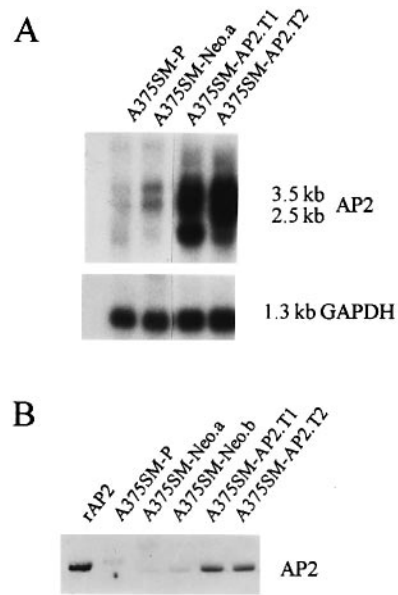


Fig. 6. (A) Northern blot analysis for the expression of AP-2 in parental A375SM, neo-transfected, and two AP-2 transfectants. High levels of AP-2 transcripts were observed in the two transfectants as compared with residual levels in parental and neo-transfected cells. The same blot was hybridized with GAPDH to verify the integrity of RNA and the amount loaded in each lane. (B) Western blot analysis for the expression of the AP-2 protein in A375SM parental, neo-transfected (neo.a and neo.b), and in the AP-2 transfectants A375SM-AP2.T1 and A375SM-AP2.T2. Note AP-2 expression was observed in the AP-2 transfectant cells, while the parental and neo-transfected cells express a very faint band. r-h-AP-2 served as a positive control. The AP-2 band in the parental cells migrated more slowly on this gel.

(wild-type) cDNA or an empty vector, neo-resistant colonies were pooled and established in culture. Two independent transfections were performed. Therefore, two transfectants were obtained and designated A375SM-AP2.T1 and A375SM-AP2.T2, respectively. Northern blot analysis using AP-2 cDNA as a probe detected high levels of AP-2 mRNA transcripts in the two transfectants (Figure 6A), but only residual levels in parental A375SM or control neo-transfected cells. Expression of AP-2 in the transfectant cells was also verified by Western blot analysis using nuclear extracts and anti-AP-2 antibody (Figure 6B). The 52 kDa protein was observed in the two transfectants as compared with faint bands in the two neo-transfected clones.

To determine whether the AP-2 in the transfectant cells is functional, we next analyzed the ability of nuclear extracts from the transfectant cells to bind to a consensus AP-2-binding DNA oligonucleotide on an EMSA gel. We used the SB-2 cells as a positive control for cells expressing the AP-2 transcription factor. As shown in Figure 7, three shifted bands were observed with nuclear extracts from SB-2 cells (lane 2), with the upper band (band 1) being super-shifted when reacted with anti-AP-2 antibody (lane 4) but not with anti-CREB antibody (lane 3). In contrast, nuclear extracts from parental A375SM cells yielded a strong band 2 and very faint bands 1 and 3 (Figure 7, lane 5), while nuclear extracts from the two AP-2 transfectants T1 and T2 yielded three strongly shifted bands similar to the pattern observed with nuclear extracts from the SB-2 cells (Figure 7, lanes 6 and 7). The upper bands (band 1) in the two transfectants were super-shifted

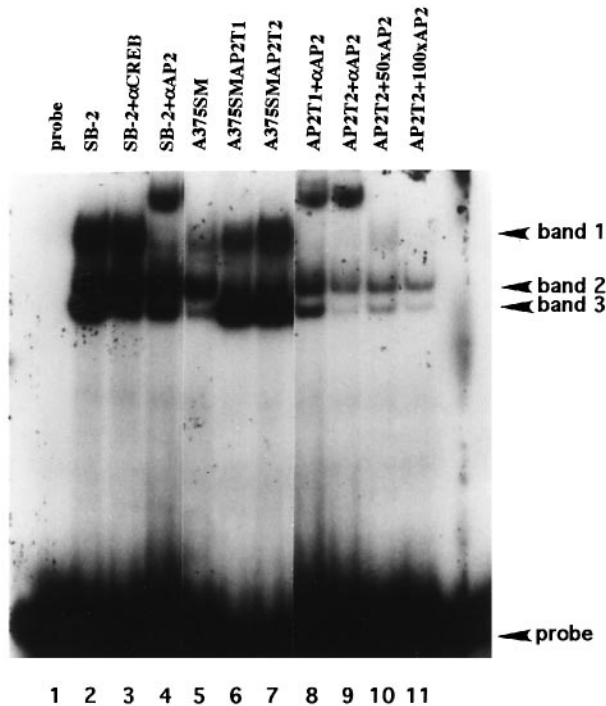


Fig. 7. EMSA gel to determine that the AP-2 in the transfected cells is functional. Nuclear extracts from control and AP-2-transfected cells were reacted with the AP-2 DNA binding motif. SB-2 cells were used as a positive control for the presence of AP-2 that yielded three shifted bands (lane 2). Band 1 was super-shifted by anti-AP-2 antibody (lane 4), but not by anti-CREB antibody (lane 3). Note AP-2 binding activity was observed in the two AP-2 transfectants A375SM-AP-2.T1 (lane 6) and A375SM-AP-2.T2 (lane 7), but not in parental A375SM cells (lane 5). Band 1 was super-shifted in the AP-2 transfected cells by anti-AP-2 antibody (lanes 8 and 9). In addition, bands 1 and 3 were competed out by an excess of unlabeled double-stranded oligonucleotide probe, while band 2 was slightly affected.

when reacted with anti-AP-2 antibody (Figure 7, lanes 8 and 9), and bands 1 and 3 were competed out using excess unlabeled double-stranded oligonucleotide probe (Figure 7, lanes 10 and 11). Band 2 was slightly competed out with $\times 100$ molar excess of unlabeled AP-2 (Figure 7, lane 11).

To further confirm that the AP-2 in the transfected cells is transcriptionally active, we took advantage of the observation that AP-2 transactivates the metallothionein promoter (Bauer *et al.*, 1994). To that end, we constructed a luciferase reporter gene expression vector driven by three AP-2 consensus response elements from the human metallothionein gene IIA ligated in front of a minimal TK promoter (Figure 8A). The reporter constructs were transfected into control or AP-2-transfected A375SM cells, together with the pB-actin-RL plasmid that served as an internal control for transfection efficiency. Luciferase activity was 5- to 8-fold higher in the AP-2 transfectants A375SM-AP-2.T1 and A375SM-AP-2.T2, respectively, as compared with the neo-control cells (Figure 8B). These data demonstrate that the two AP-2 stably transfected T1 and T2 clones expressed high levels of active AP-2.

Upregulation of c-KIT gene expression in AP-2 stably transfected human melanoma cells

Our promoter analyses (Figure 3A) indicated that the AP-2 transcription factor is an important regulator of

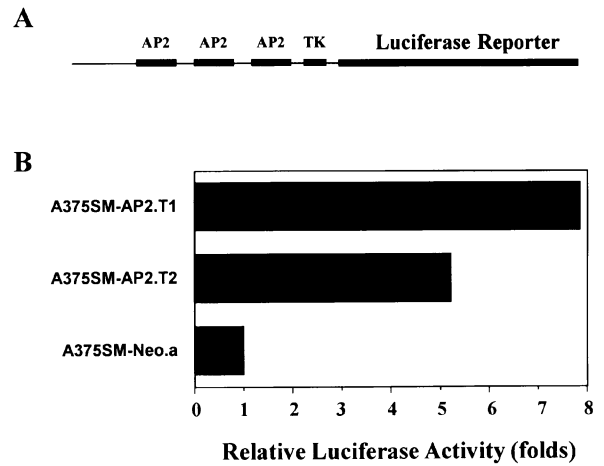


Fig. 8. (A) Construction of the luciferase reporter gene (3XAP2-Luc) driven by a trimer of AP-2 binding motifs and minimal TK promoter. (B) Luciferase activity of 3XAP2-Luc in A375SM neo and AP-2 transfectants. Melanoma cells (2×10^5) were co-transfected with 1 μ g of 3XAP2-Luc and 0.05 μ g of pB-Actin-RL plasmid. Firefly and Renilla luciferase activities were measured by the Dual-Luciferase Reporter Assay System. Note luciferase activity was upregulated in the two AP-2 transfected clones but not in neo-transfected cells, which demonstrates that the transfected AP-2 was functional.

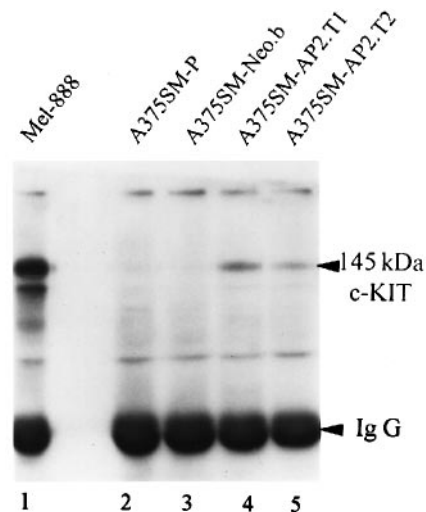


Fig. 9. Upregulation of c-KIT gene expression in AP-2 stably transfected human melanoma cells. c-KIT expression was determined by immunoprecipitation plus Western blot analysis. Re-expression of the 145 kDa c-KIT receptor was observed in the AP-2-transfected cells (lanes 4 and 5) but not in parental (lane 2) or neo-transfected cells (lane 3). The human melanoma cell line Mel-888 served as a positive control for the expression of c-KIT (lane 1).

c-KIT gene expression. We therefore examined the effect of AP-2 re-expression in A375SM cells on c-KIT gene expression. To that end, the expression of the 145 kDa c-KIT receptor was analyzed in the two AP-2 transfectants, T1 and T2. To determine c-KIT protein expression, whole-cell lysates were prepared and reacted with specific polyclonal anti-c-KIT antibody, the immunocomplexes were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and c-KIT expression was determined by Western blot analysis. Figure 9 shows that c-KIT was expressed in the two AP-2 transfectants A375SM-AP-2.T1 and A375SM-AP-2.T2 but not in parental or control neo-transfected cells. We used the

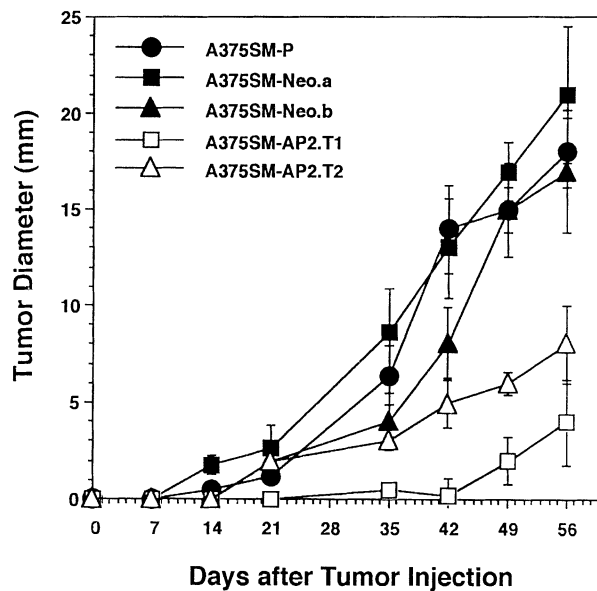


Fig. 10. Tumorigenicity of A375SM parental, neo-transfected and AP-2-transfected cells, 60 days after s.c. injection into nude mice. Tumor cells (1×10^6 in 0.2 ml of HBSS) were injected over the right scapular region. Growth of s.c. tumors were monitored by weekly examination and measurement of tumors with calipers. The data represent the mean diameter observed in five animals per group.

Mel-888 cells as a positive control for *c-KIT* gene expression (Figure 9, lane 1). These results were confirmed by RT-PCR (data not shown).

Tumorigenicity and metastatic potential of AP-2 transfected human melanoma cells

We have previously demonstrated that enforced *c-KIT* expression significantly inhibited tumor growth and metastasis of A375SM human melanoma cells (Huang *et al.*, 1996). To determine the tumorigenicity of the AP-2-transfected A375SM cells which exhibited increased expression of *c-KIT*, we injected 1×10^6 cells s.c. into BALB/c nude mice and tumor growth was monitored once a week for 60 days. Both A375SM parental and neo-transfected control cells grew in all mice (100% tumor uptake) and reached 1.0–1.4 cm in mean diameter within 6 weeks (Figure 10). In contrast, A375SM AP-2-transfected cells did not begin to form palpable tumors until 3 weeks (T2) or 7 weeks (T1) after injection, and produced smaller tumors (0–4.5 mm in mean diameter). Growth of the A375SM-AP-2.T1 cells differed significantly from parental and neo-control cells at all times ($p < 0.01$), while the difference between the A375SM-AP2.T2 and parental or neo-transfected cells was statistically significant 5 and 6 weeks after injection ($p < 0.05$) (Figure 10). These data suggest that transfection of AP-2 into A375SM cells suppressed their tumor growth *in vivo*.

In the next set of experiments, the metastatic potential of AP-2-transfected A375SM cells was determined in an experimental lung metastasis assay (Radinsky *et al.*, 1994; Xie *et al.*, 1997b). To that end, BALB/c nude mice were injected i.v. with 1×10^6 A375SM AP-2-transfected, parental, or neo-control cells, and 60 days later the number of lung metastases was counted. As shown in Table I, A375SM and A375SM-Neo (Neo.a and Neo.b, respectively) produced a high number of lung tumor colonies

Table I. Metastatic potential of A375SM human melanoma cells transfected with transcription factor AP-2

Cell lines	Experimental lung metastasis ^a			
	Incidence	Median	Range	p^b
A375SM-P	5/5	121	74–>200	–
A375SM-Neo.a	5/5	153	111–>200	>0.05
A375SM-Neo.b	5/5	87	54–143	>0.05
A375SM-AP-2.T1	0/5	0	all 0	>0.01
A375SM-AP-2.T2	3/5	19	0–47	<0.05

^aTumor cells (1×10^6 /mouse) were injected i.v. into nude mice; experimental metastasis was determined 60 days after tumor injection. ^bThe differences in metastatic potential between parental cells and transfected cells were analyzed by the Mann–Whitney U test.

in all injected mice. In contrast, the A375SM-AP-2-transfected cells did not metastasize to the lungs (T1) or produced a few lung metastases in some mice (T2). To determine whether those metastatic revertants still overexpressed AP-2, five metastatic nodules were isolated and established as individual cell lines. AP-2 expression in these cell lines was analyzed by Northern blot. We could not detect increased levels of AP-2 mRNA in these cell lines as compared with parental and neo-control cells (data not shown). These data implied that the occasional gain of metastatic potential in A375SM-AP-2 cells was due to the loss of exogenous AP-2 expression. Alternatively, the pooled transfectant cells may contain some neo-resistant but AP-2-negative cells. In this case, the AP-2-negative nodes could represent a selection of these cells rather than ‘revertants’.

To determine whether the inhibition of tumor growth and the decreased capacity to produce lung metastases by A375SM-AP2 cells *in vivo* was due to different growth rates *in vitro*, we compared their doubling time with control cells in cultures. When the cells were cultured in medium containing 0.5% fetal bovine serum (FBS) (a limiting condition), the cell doubling times of A375SM parental and neo-control cells were indistinguishable from the A375SM-AP-2 cells (45 h). In contrast, in medium containing 10% FBS, the doubling time for parental and neo-transfected cells was 24 h while the AP-2-transfected cells doubled every 36 h. In addition, in medium containing 10% FBS, the AP-2-transfected cells exhibited morphological changes: they were enlarged and flat, and had an increased number of multinucleated cells (data not shown). These later effects could be due to the presence of SCF in the serum (see below).

Induction of apoptosis in AP-2-transfected melanoma cells by SCF

Previous studies from our laboratory have demonstrated that SCF induces apoptosis in *c-KIT*-expressing human melanoma cells under both *in vitro* and *in vivo* conditions (Huang *et al.*, 1996). Therefore, we next determined whether expression of *c-KIT* in AP-2-transfected A375SM cells is associated with apoptosis. A375SM-AP2.T2 and A375SM-neo cells were treated with 150 ng/ml human recombinant SCF in 0.5% FBS-containing medium for 72 and 96 h. Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) staining of DNA fragmentation. The frequency

of apoptosis in A375SM-AP2.T2 cells after 72 h was $17 \pm 3\%$ versus $3 \pm 0.4\%$ for neo-control A375SM cells in medium with SCF (mean \pm SEM, $p < 0.01$). After 96 h, SCF induced apoptosis of $27 \pm 4\%$ in A375SM-AP2.T2 cells as compared with $4 \pm 1\%$ in control cells. The frequency of apoptosis in A375SM-AP2.T2 cells was $5 \pm 0.5\%$ versus $2 \pm 0.4\%$ for neo-control cells in medium lacking SCF (mean \pm SEM, $p < 0.05$). These results demonstrate that SCF treatment resulted in marked induction of apoptosis in AP-2-transfected A375SM cells, which can be explained by transactivation of *c-KIT* gene expression by the AP-2 transcription factor. As apoptosis of melanoma cells expressing *c-KIT* also occurs *in vivo* due to the secretion of SCF by the host (Huang *et al.*, 1996), this is a potential mechanism by which transfection of melanoma cells with the AP-2 transcription factor leads to inhibition of tumor growth and metastasis.

Discussion

In this paper we demonstrate for the first time that the transcription factor AP-2 plays a major role in regulating tumor growth and metastasis of human melanoma. Loss of AP-2 results in downregulation of *c-KIT*, enhancement of melanoma tumorigenicity and metastasis, and failure of SCF to induce apoptosis.

c-KIT is a tyrosine-kinase type receptor whose expression progressively decreases during local tumor growth and invasion of human melanomas (Lassam and Bickford, 1992; Natali *et al.*, 1992; Zakut *et al.*, 1993). Lack of *c-KIT* expression directly correlates with the metastatic potential of human melanoma cells in nude mice (Gutman *et al.*, 1994). Ectopic expression of *c-KIT* in highly metastatic cells inhibited their tumor growth and metastatic potential *in vivo*. In addition, we found that exposure of *c-KIT*-positive melanoma cells to SCF, the ligand for *c-KIT*, under both *in vitro* and *in vivo* conditions, triggered apoptosis of these cells but not of normal melanocytes (Huang *et al.*, 1996), thus providing a mechanism by which suppression of *c-KIT* expression could contribute to the metastatic phenotype of human melanoma. The mechanism by which malignant melanoma cells lose *c-KIT* expression was unclear. Here we provide evidence for the first time that in metastatic melanoma cells, lack of *c-KIT* expression correlates with lack of expression of the AP-2 transcription factor, and that in melanoma cells AP-2 serves as a positive regulator of *c-KIT* expression. Indeed, re-expression of AP-2 in the highly metastatic A375SM cells resulted in re-expression of the *c-KIT* receptor and in turn inhibition of tumor growth and metastasis.

Upregulation of *c-KIT* expression in the AP-2-transfected cells might be one of the mechanisms (via apoptosis) for the reduction in tumorigenicity and the ability of these cells to form lung metastases *in vivo*. However, in other studies we have demonstrated that the adhesion molecule MCAM/MUC18 that plays a major role in the acquisition of the metastatic phenotype in human melanoma (Lehmann *et al.*, 1989; Luca *et al.*, 1993; Xie *et al.*, 1997a) is also regulated by the AP-2 transcription factor (Jean *et al.*, 1998). In these studies we showed that loss of AP-2 resulted in upregulation of MCAM/MUC18 and an increase in tumor growth and metastasis of human melanoma

cells. The MCAM/MUC18 promoter contains four AP-2 binding sites and its activation is regulated by AP-2, thus representing a second example of 'melanoma antigen' to be regulated by AP-2 (Jean *et al.*, 1998). In addition, other genes that are involved in the progression of human melanoma such as MMP-2, the metalloproteinase that degrades collagen type IV (Luca *et al.*, 1997), E-cadherin (Cowley and Smith, 1996), p21/WAF-1 (Jiang *et al.*, 1995; Vidal *et al.*, 1995), HER-2 (Natali *et al.*, 1994) and *bcl-2* (Van den Oord *et al.*, 1994; Grover and Wilson, 1996) have either already been shown to be regulated by AP-2 (Bosher *et al.*, 1995; Zeng *et al.*, 1997) or represent likely targets for AP-2 gene regulation based on the existence of AP-2 elements in their promoters. Therefore, we propose that loss of AP-2 expression may be the crucial event in the development of malignant melanoma.

The mechanisms accounting for lack of expression of AP-2 or downregulation in malignant melanoma cells are currently unknown. One tempting possibility is to link the lack of AP-2 expression to cytogenetic evidence demonstrating that the majority of malignant melanoma cells exhibit deletion of the distal portion of the long arm of chromosome 6 (Pathak *et al.*, 1983; Trent *et al.*, 1983; Copeman, 1992; Healy *et al.*, 1995; Zhang *et al.*, 1995) or more importantly with abnormalities in the short arm of chromosome 6 (6p), near the HLA locus to which the AP-2 gene is mapped (Gaynor *et al.*, 1991). Indeed, deletions and loss of heterozygosity (LOH) have been reported in this region in malignant melanoma (Pathak *et al.*, 1983; Walker *et al.*, 1995). Moreover, re-introduction of chromosome 6 into metastatic melanoma cells inhibited their tumorigenicity and metastatic potential (Trent *et al.*, 1990; Welch *et al.*, 1994). These studies suggest that inactivation of a tumor suppressor gene on chromosome 6 may be the critical event in the progression of melanoma. The mechanism for the lack of AP-2 expression in the metastatic melanoma cells used in our study is currently under investigation.

Epidemiological data suggest that exposure to UV radiation plays a major role in the development of at least some cutaneous melanoma (Halman *et al.*, 1980; Romerdhal *et al.*, 1988). The role of UV radiation in the development of human cutaneous melanoma to its metastatic state is not very well characterized. We have recently demonstrated that UV-B irradiation can promote tumorigenic and metastatic properties in primary cutaneous melanoma via induction of interleukin-8 (Singh *et al.*, 1995; Luca *et al.*, 1997). A provocative idea will be to link abnormalities in the AP-2 gene with UV radiation that serves as a carcinogen for cutaneous melanoma. Indeed, two recent reports have shown that both UV-A and UV-B radiation can alter the expression and activity of the AP-2 transcription factor in human keratinocytes and epidermoid carcinoma cell line (A431) (Ariizumi *et al.*, 1996; Grether-Beck *et al.*, 1996). The question of whether UV radiation can effect AP-2 expression and activity in human primary cutaneous melanoma is currently under investigation in our laboratory.

Despite improvement in early detection and treatment of primary melanoma, the diagnosis of melanoma is often made after the cancer has already metastasized to the regional and distant lymph nodes, liver, lung and

central nervous system (Fidler, 1992). Since current staging systems enable us to identify only some of the melanoma patients who will develop metastases, better prognostic determinants need to be identified. Clearly, AP-2 falls within this category. Our analysis of AP-2 expression in human melanoma cell lines (although limited to a small number of melanoma cell lines tested) indicates that AP-2 expression was observed only in cell lines derived from primary cutaneous melanoma (radial growth phase) such as SB-2 and Mel-501 (both cell lines are low-tumorigenic and non-metastatic in nude mice), but not in cell lines derived from metastatic lesions. It will be extremely important to determine at what stage in the transformation process from melanocytes to malignant melanoma this event occurs. Currently, several hundred human melanoma specimens gathered at the M.D.Anderson Cancer Center representing different stages in the progression of human melanoma (from atypical nevi to metastatic lesions) are being evaluated for AP-2 expression. Our aim is to establish the validity of using the AP-2 protein as a prognostic marker and eventually as a common target for anti-tumor and anti-metastatic therapy.

Materials and methods

Cell lines and culture conditions

The A375-P human melanoma cell-line was established in culture from a lymph node metastasis of a melanoma patient (Kozlowski *et al.*, 1984). The highly metastatic derivative A375SM was established from pooled lung metastases produced by the A375-P cells injected i.v. into nude mice (Li *et al.*, 1989). The SB-2 cell line was isolated from a primary cutaneous lesion and was a gift of Dr B.Giovanella (St Joseph's Hospital Cancer Center, Research Laboratory, Houston, TX). In nude mice, SB-2 cells are poorly tumorigenic and nonmetastatic (Luca *et al.*, 1993; Singh *et al.*, 1995). SB-3 was isolated from a cutaneous metastasis and was found to be with non to low metastatic potential in nude mice (Luca *et al.*, 1995). The human melanoma cell line WM-2664 was purchased from ATCC, and is highly metastatic in nude mice (Luca *et al.*, 1995). Mel-501 and Mel-888 cell lines were provided by Dr Ruth Halaban (Yale University School of Medicine, New Haven, CT). In nude mice, Mel-501 and Mel-888 are low metastatic. The MeWo cell line was kindly provided to us by Dr Z.Ronai (Mount Sinai, NY). In nude mice MeWo cells have low to intermediate metastatic potential.

The melanoma cell lines were maintained in culture as adherent monolayers in Eagle's minimal essential medium (MEM) supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, two-fold vitamin solution and penicillin-streptomycin (Flow Laboratories, Rockville, MD), and incubated in 5% CO₂, 95% air at 37°C.

Animals

Male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 8 weeks of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health.

Plasmid constructs

The *c-KIT* promoter luciferase plasmid (pKluc) was generated by PCR amplification of the 5'-flanking region of the *c-KIT* gene (-1 to -1215 nucleotides). The adenosine in the ATG codon was designated +1. Nucleotides upstream from this site were assigned negative numbers. The PCR product was subcloned upstream of the firefly luciferase gene by using *HindIII* and *KpnI* sites. The sequence of the promoter upstream of the luciferase reporter was verified. The pB-Actin-RL was generated by digestion of pBluescript B-Actin plasmid (kindly provided by Dr Bing Su at M.D.Anderson Cancer Center) with *XhoI-HindIII*, and

subcloning the β -actin promoter upstream of the Renilla luciferase reporter gene by ligation into *XhoI-HindIII*-digested pRL-null (Promega Corp., Madison, WI). The AP-2 binding site luciferase reporter (3XAP-2-Luciferase) was generated by digestion of 3XAP2 CAT-reporter plasmid (Buettner *et al.*, 1993; Kannan *et al.*, 1994) with *HindIII* and *KpnI* sites. The sequence of the promoter upstream of the luciferase reporter was verified. The AP-2 expression plasmids pSG5-AP-2 and pSG5-Neo-AP-2, their control vectors pSG5 and pSG5-Neo, and the AP-2B expression plasmid pSG-5-Neo-AP-2B were described previously (Kannan *et al.*, 1994).

Mutagenesis

The mutants Mut.1-pKluc and Mut.2-pKluc were generated from the pKluc vector using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The following oligonucleotides were used as primers: 5'-GCGAGGAGGGGTTTGGTTGCGCGCAGAGGGAGG-3' and 5'-CCTCCCTGCGCGCAAGCCAAACCCCTCTCGC-3' for the mutant Mut.1-pKluc, and 5'-CGCCGCTCGCGGTTCTGGTTGCTCGGCTTGC-3' and 5'-GCAAAGCCGAGCAACCAGAACCAGCG-AGCGGCG-3' for the mutant Mut.2-pKluc. The presence of mutations were verified by sequencing with the primer 5'-CGGCATTAAC-ACGTC-3'.

Transient transfections and luciferase assays

Melanoma cells (2×10^5) were transfected by using the Lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD). For the experiments shown in Figure 1, 2 μ g of pKluc and 0.05 μ g pB-Actin-RL-plasmids were used. For the experiments shown in Figure 3A, 2 μ g of pKluc, 0.05 μ g of pB-Actin RL plasmid, and 1–5 μ g of pSG5-AP-2 expression plasmids were used. For the experiments shown in Figure 3B, 1 μ g of pKluc and 0.05 μ g of pB-Actin-RL plasmids were used. At 10 h post-transfection, the medium was changed to serum-containing complete medium and the cells were incubated further for 48 h at 37°C. The cells were then washed with phosphate-buffered saline (PBS) and harvested in Passive Lysis Buffer (Promega Corp., Madison, WI). Quantitation of firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI). Normalization of transfection efficiency was done using a co-transfected β -actin-Renilla luciferase expression vector. Fold activation or inhibition of luciferase activity was calculated relative to control cells which were given the reference value of 1 as described by Janknecht and Hunter (1997).

Stable transfection of melanoma cells with AP-2

5×10^6 A375SM cells were transfected using 15 μ l of lipofectin reagent (Life Technologies, Inc. Gaithersburg, MD) and 2 μ g of pSG5-Neo-AP-2 expression vector or control pSG5-Neo vector. Transfections were carried out according to the manufacturer's instructions. At 10 h post-transfection, the medium was changed to serum-containing complete medium and the cells were further incubated for 48 h at 37°C. Cells were selected after 48 h with standard medium containing G418 500 μ g/ml. Fourteen days later, neo-resistant colonies were isolated by trypsinization and established in culture.

Northern blot analysis

mRNA was extracted from 10^6 – 10^7 cultured cells using the FastTrack™ KIT (Invitrogen Co., San Diego, CA). For Northern blot analysis, 2.5 μ g of purified mRNA was separated on 1% denaturing formaldehyde/agarose gels, electrotransferred at 0.6 A to GeneScreen® nylon membrane (DuPont Co., Boston, MA) and UV cross-linked with 120 000 μ J/cm² using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Hybridizations were performed as previously described (Luca *et al.*, 1993, 1995) and filters were washed three times with 30 mM NaCl/3 mM sodium citrate pH 7.2/sodium dodecyl sulfate (0.1% w/v at 60°C).

The DNA probes used in these analyses were a 1.2 kb *SaI* fragment of the *c-KIT* gene (phc-KIT-171, American Type Culture Collection; Yarden *et al.*, 1987), a 1.9 kb AP-2 cDNA fragment digested with *EcoRI* from the pNAP2 plasmid (Kannan *et al.*, 1994), and a 1.3 kb *PstI* cDNA fragment corresponding to rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fort *et al.*, 1985). Each DNA fragment was purified by agarose gel electrophoresis, recovered using Gene Clean (BIO 101, La Jolla, CA), and radiolabeled by the random primer technique using [α -³²P]deoxyribonucleotide triphosphates.

Western blot

Melanoma cell nuclei were isolated as described previously (Hudson *et al.*, 1995). Isolated nuclei were lysed by using Triton X-100 lysis buffer [150 mM NaCl, 25 mM Tris pH 7.5, 1% (w/v) Triton X-100,

2 mM EDTA, 1 mM PMSF, 10 µg/ml trypsin inhibitor, 20 mM leupeptin, 0.15 U/ml aprotinin] on ice for 20 min. The soluble protein in the lysates was then separated by centrifugation at 14 000 r.p.m. for 20 min at 4°C. Protein concentration was assayed by the Bio-Rad protein assay reagent (Bio-Rad Laboratories) and stored at -70°C. Before loading, protein samples were boiled in sample buffer [62.5 mM Tris-HCl pH 6.8, 10% (w/v) glycerol, 100 mM DTT, 2.3% SDS, 0.002% bromophenol blue] for 2–5 min and cooled on ice for 5–10 min. Samples (30 µl) were loaded and separated on 10% SDS-PAGE at 150–200 V for 45–60 min in electrophoresis buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS). Proteins in the gels were electrophoretically transferred onto immobilon-P transfer membrane (Millipore) in 1× transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol) at 100 V for 1 h at 4°C. The membranes were washed in blocking buffer [TBS (10 mM Tris-HCl pH 8.0, 150 mM NaCl) and 3% BSA, 1% OVA, 0.02% NaN₃] for 2 h at room temperature (RT) with shaking, and then rinsed once briefly with TTBS (99.9% TBS, 0.1% Tween 20). The membrane was incubated in diluted 1:500 anti-AP-2 polyclonal antibody (C-18, Santa Cruz Biotechnology) overnight at 4°C. The membranes were rinsed twice briefly with TTBS and washed three times with TBS at RT, and then incubated with a dilution of 1:5000 second antibody (anti-rabbit immunoglobulin, horseradish peroxidase-linked F(ab)₂ fragment from donkey) for 1 h at RT with shaking. The membranes were rinsed twice briefly and washed three times with TBS at RT with shaking. The probe proteins were detected with Amersham ECL system according to the manufacturer's instructions.

Electrophoretic mobility gel shift assay (EMSA)

The double-stranded DNA fragment corresponding to *c-KIT* promoter region of -68 to -175 was amplified by PCR. *c-KIT* promoter luciferase reporter plasmid served as the template. The volume was adjusted to 50 µl by adding 200 ng of DNA, 35 µl of PCR buffer (10× concentrated; 500 mM KCl, 100 mM Tris, pH 8.3), 2 µl of 25 mM MgCl₂, 0.5 µl each two primers (15 µM), 1 µl each of dATP, dCTP, dGTP, and dTTP, (2.5 mM), and 0.5 U of *Taq* DNA polymerase. The PCR amplification was carried out for 30 cycles in a DNA thermal cycler (Perkin-Elmer/Cetus). Each cycle included denaturation at 94°C for 1 min, re-annealing of primers at 55°C for 1 min and extension at 72°C for 1.5 min. The sequence structures of the primers are: *c-KIT* primer F 5'-GGCGCG-AGGGAGGGGAGGCGAGGA-3' and *c-KIT* primer R 5'-AAGTGCA-GCGAGCGCGCAAAGCC-5'. The 108 bp PCR product was fractionated on electrophoresed agarose gel and isolated by using Gene Clean kit (Bio 101) and used in EMSA. Nuclear protein extracts were assayed for protein content by the Bio-Rad protein assay reagent (Bio-Rad Laboratories) and stored at -70°C. Gel shift assays were performed based on the method of Carthew *et al.* (1985). DNA reactions were performed in a volume of 25 µl with 1 µg of poly(dI-dC), 2 µg BSA, and 12% glycerol in HEPES buffer (pH 7.9). Ten micrograms of nuclear extracts and 30 000 c.p.m. of end-labeled double-stranded DNA probe were added to the reaction mixture. The binding reactions were left on ice for 30 min. Protein-DNA complexes were resolved on a 6% non-denaturing polyacrylamide gel. The gels were dried and exposed to X-ray film at -80°C overnight. Human recombinant AP-2 protein was purchased from Promega. For competition assays, unlabeled oligonucleotides were added 10 min before adding the labeled probes. Wild-type or mutant AP-2 oligonucleotides were purchased from Santa-Cruz. The wild-type AP-2 binding motif 5'-GAT CGA ACT GAC CGC CCG CGG CCC CT-3' was used as a probe in the EMSA gels. In the mutated AP-2 oligonucleotides, the underlined CC were changed to TT. Anti-AP-2 antibodies (Santa-Cruz, CA) were used in the supershift analyses.

Immunoprecipitation

Melanoma cells (1.5–2.5×10⁶ in 10 ml of MEM) before and after transfection were seeded in 100 mm Petri dishes and incubated overnight. The cells were scraped off and washed in 4°C PBS containing 5 mM EDTA. The cell pellets were lysed into 100 µl Triton X-100 lysis buffer and kept on ice for 20 min. The soluble protein in the lysates was then separated by centrifugation at 14 000 r.p.m. for 20 min at 4°C, and aliquoted and stored at -70°C until use. Protein concentration in the lysates was determined by the Bio-Rad protein assay reagent (Bio-Rad Laboratories). The A375SM cell lysates were adjusted to the same volume (1 ml) and same protein concentration (2.5 mg) by using Triton lysis buffer. Mel-888 cell lysate was adjusted to 1 ml volume and 30 µg protein by lysis buffer as above. Cell lysates were incubated with 25 µl human anti-c-KIT polyclonal antibody (kindly provided by Dr Keith Langley, Amgen, Thousand Oaks, CA) for 4 h at 4°C and then added 50 µl of protein A-agarose for overnight incubation with rotation. The

pellets were washed three times and boiled with sample buffer. The supernatants were loaded onto and separated on 7.5% SDS-PAGE. The Western blot analysis was performed as described above by using the same anti-c-KIT antibody.

Tumor cell injections

To prepare tumor cells for inoculation, cells in exponential growth phase were harvested by brief exposure to 0.25% trypsin + 0.2% EDTA solution (w/v). The flask was sharply tapped to dislodge the cells, and supplemented medium was added. The cell suspension was pipetted to produce a single-cell suspension. The cells were washed and resuspended in Ca²⁺- and Mg²⁺-free HBSS to the desired cell concentration. Cell viability was determined by Trypan-Blue exclusion, and only single-cell suspensions of 90% viability were used. Tumors (s.c.) were produced by injecting 1×10⁶ tumor cells in 0.2 ml HBSS over the right scapular region. Growth of s.c. tumors was monitored by examination of the mice every day and weekly measurement of tumors with calipers. The mice were killed 2 months after injection, and tumors were processed for hematoxylin and eosin staining.

For experimental lung metastasis 1×10⁶ tumor cells in 0.2 ml of HBSS were injected into the lateral tail vein of nude mice. The mice were killed after 60 days, and the lungs were removed, washed in water, and fixed with Bouin's solution for 24 h to facilitate counting of tumor nodules as described previously (Radinsky *et al.*, 1994; Huang *et al.*, 1996). The number of surface tumor nodules was counted under a dissecting microscope. Sections of the lungs were stained with hematoxylin and eosin to confirm that the nodules were melanoma and to identify micrometastasis.

Terminal deoxynucleotidyl transferase-mediated nick end-labeling method for *in situ* detection of apoptotic cells

The TUNEL method (Gavrieli *et al.*, 1992) was used to identify DNA fragmentation in cells undergoing apoptosis as previously described (Radinsky *et al.*, 1994). Briefly, nuclei on slides with cell cultures were stripped from proteins by incubation with 20 µg/ml proteinase K for 15 min at RT, and the slides were then washed four times in double-distilled water (ddH₂O) for 2 min. Endogenous peroxidase was inactivated by covering the sections with 2% H₂O₂ for 5 min at RT. The sections were rinsed with DDW and immersed in TDT buffer (30 mM Tris-HCl pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TDT (0.3 µl/µl) and biotinylated dUTP in TDT buffer were then added to cover the sections and then incubated in a humidified atmosphere at 37°C for 60 min. The sections was terminated by transferring the slides to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at RT. The sections were rinsed with ddH₂O, covered with 2% aqueous solution of BSA for 10 min at RT, rinsed in ddH₂O, and immersed in PBS for 5 min. The sections were covered with extravidin peroxidase (BioMakor, Rehovot, Israel), diluted 1:10–20 in water, incubated for 30 min at 37°C, washed in ddH₂O, immersed for 5 min in PBS and stained with AEC for ~30 min at 37°C.

Statistics

The significance of the *in vitro* results was determined by the Student's *t*-test (two-tailed); the significance of the *in vivo* metastasis results was determined by the Mann-Whitney U-test.

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References

- Ariizumi,K., Bergstresser,P.R. and Takashima,A. (1996) Wavelength-specific induction of immediate early genes by ultraviolet radiation. *J. Dermatol. Sci.*, **12**, 147–155.
- Bar-Eli,M. (1997) Molecular mechanisms of melanoma metastasis. *J. Cell Physiol.*, **173**, 275–278.
- Bauer,R., Imhof,A., Pscherer,A., Kopp,H., Moser,M., Seagers,S., Kerschmer,M., Tainsky,M.A., Hofstaedter,F. and Buettner,R. (1994) The genomic structure of the human AP-2 transcription factor. *Nucleic Acids Res.*, **22**, 1413–1420.

- Bosher, J.M., Williams, T. and Hurst, H.C. (1995) The developmentally regulated transcription factor AP-2 is involved in *c-erbB-2* overexpression in human mammary carcinoma. *Proc. Natl Acad. Sci. USA*, **92**, 744–747.
- Brannan, C.I., Lyman, S.D., Williams, D.E., Eisenman, J., Anderson, D., Cosman, D., Bedell, M.A., Jenkins, N.A. and Coopeland, N.G. (1991) Steel-Dickie mutation encodes a c-KIT ligand lacking transmembrane and cytoplasmic domains. *Proc. Natl Acad. Sci. USA*, **88**, 4671–4674.
- Buettner, R., Kannan, P., Imhof, A., Bauer, R., Yim, S.O., Glackshuber, R., van Dyke, M.W. and Tainsky, M.A. (1993) An alternatively spliced mRNA from the AP-2 gene encodes a negative regulator of transcriptional activation of AP-2. *Mol. Cell. Biol.*, **13**, 4174–4185.
- Carthew, R.W., Codosh, L.A. and Sharp, P.A. (1985) An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. *Cell*, **43**, 439–448.
- Chabot, B., Stephenson, D.A., Chapman, V.M., Besmer, P. and Bernstein, A. (1988) The protooncogene c-kit encoding a trans-membrane tyrosine kinase receptor maps to the mouse *W* locus. *Nature*, **335**, 88–89.
- Copeman, M.C. (1992) The putative melanoma tumor-suppressor gene on human chromosome 6q. *Pathology*, **24**, 307–309.
- Cowley, G.P. and Smith, M.E. (1996) Cadherin expression in melanocytic naevi and malignant melanomas. *Am. J. Pathol.*, **179**, 183–187.
- Descheemaeker, K.A., Wyns, S., Nelles, L., Auwerx, J., Ny, T. and Collen, D. (1992) Interaction of AP-1-like, AP-2-like and SP-1-like proteins with two distinct sites in the upstream regulatory region of the plasminogen activator inhibitor-1 gene mediates the phorbol 12-myristate 13-acetate response. *J. Biol. Chem.*, **267**, 15086–15091.
- Duan, C. and Clemmons, D.R. (1995) Transcription factor AP-2 regulates human insulin-like growth factor binding protein-5 gene expression. *J. Biol. Chem.*, **270**, 24844–24851.
- Fidler, I.J. (1990) Critical factors in the biology of human cancer metastasis. *Cancer Res.*, **50**, 6130–6138.
- Fidler, I.J. (1992) The biology of melanoma metastasis. In Balch, C.M., Houghton, A.N., Milton, G.W., Sober, A.J. and Soong, S.J. (eds) *Cutaneous Melanoma*. J.B. Lippincott Company, Philadelphia, PA, pp. 112–129.
- Flanagan, J.G., Chan, D.G. and Leder, P. (1991) Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the *sl^d* mutant. *Cell*, **64**, 1025–1035.
- Fleischman, R.A. (1992) Human piebald trait resulting from a dominant negative mutant allele of the c-KIT membrane receptor gene. *J. Clin. Invest.*, **89**, 1713–1717.
- Fleischman, R.A., Saltman, K.L., Stastny, V. and Zneimer, S. (1991) Deletion of the c-kit protooncogene in the human development defect piebald trait. *Proc. Natl Acad. Sci. USA*, **88**, 10885–10889.
- Fort, P., Marty, L., Piechaczyk, M., Sabrouy, S.E., Dani, C., Jeanteur, P. and Blanchard, J.M. (1985) Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res.*, **13**, 1431–1442.
- Gavrieli, Y., Sherman, Y. and Ben-Sasson, S.A. (1992) Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation. *J. Cell Biol.*, **119**, 493–501.
- Gaynor, R.B., Muchardt, C., Xia, Y.R., Klisak, I., Mohandas, T., Sparkes, R.S. and Luskis, A.J. (1991) Localization of the gene for the DNA-binding protein AP-2 to human chromosome 6p 22.3-pter. *Genomics*, **10**, 1100–1102.
- Geisler, E.N., Ryan, M.A. and Housman, D.E. (1988) The dominant white spotting (*W*) locus of the mouse encodes the c-KIT protooncogene. *Cell*, **55**, 185–192.
- Giebel, L.B. and Spritz, R.A. (1991) Mutation of the KIT (mast/stem cell growth factor receptor) proto-oncogene in human piebaldism. *Proc. Natl Acad. Sci. USA*, **88**, 8696–8699.
- Grether-Beck, S., Olaizola-Horn, S., Schmitt, H., Grewe, M., Jahnke, A., Johnson, J.P., Briviba, K., Sies, H. and Krutmann, J. (1996) Activation of transcription factor AP-2 mediates UVA radiation- and singlet oxygen-induced expression of the human intercellular adhesion molecule gene. *Proc. Natl Acad. Sci. USA*, **93**, 14586–14591.
- Grover, R. and Wilson, G.D. (1996) Bcl-2 expression in malignant melanoma. *Eur. J. Surg. Oncol.*, **22**, 347–349.
- Gutman, M., Singh, R.K., Radinsky, R. and Bar-Eli, M. (1994) Intertumoral heterogeneity of receptor-tyrosine kinases expression in human melanoma cell lines with different metastatic capabilities. *Anticancer Res.*, **14**, 1759–1766.
- Haslinger, A. and Karin, M. (1985) Upstream promoter element of the human metallothionein-IIA gene can act like an enhancer element. *Proc. Natl Acad. Sci. USA*, **82**, 8572–8576.
- Healy, E., Rehman, I., Angus, B. and Rees, J.L. (1995) Loss of heterozygosity in sporadic primary cutaneous melanoma. *Genes Chromosomes. Cancer*, **12**, 152–156.
- Holman, C.D.J., Mulroney, C.D. and Armstrong, B.K. (1980) Epidemiology of preinvasive and invasive malignant melanoma in Western Australia. *Int. J. Cancer*, **25**, 317–323.
- Huang, S., Luca, M., Gutman, M., McConkey, D.J., Langley, K.E., Lyman, S.D. and Bar-Eli, M. (1996) Enforced c-KIT expression renders highly metastatic human melanoma cells susceptible to stem cell factor-induced apoptosis and inhibits their tumorigenic and metastatic potential. *Oncogene*, **13**, 2339–2347.
- Hudson, J.M., Frade, R. and Bar-Eli, M. (1995) Wild-type p53 regulates its own transcription in a cell-type-specific manner. *DNA Cell Biol.*, **14**, 759–766.
- Hyman, S.E., Comb, M., Pearlberg, J. and Goodman, H.M. (1989) An AP-2 element acts synergistically with the cyclic AMP and phorbol ester-inducible enhancer of the human proenkephalin gene. *Mol. Cell. Biol.*, **9**, 321–324.
- Janknecht, R. and Hunter, T. (1997) Convergence of MAP-kinase pathways on the ternary complex factor Sap-1a. *EMBO J.*, **16**, 1620–1627.
- Jean, D., Gershenwald, J.E., Huang, S., Luca, M., Hudson, M.J., Tainsky, A.M. and Bar-Eli, M. (1998) Loss of AP-2 results in upregulation of MCAM/MUC18 and an increase in tumor growth and metastasis of human melanoma cells. *J. Biol. Chem.*, **273**, 16501–16508.
- Jiang, H., Lin, J., Su, Z.Z., Herlyn, M., Kerbel, R.S., Weissman, B.E., Welch, D.R. and Fisher, P.B. (1995) The melanoma differentiation-associated gene *mda-6*, which encodes the cyclin-dependent kinase inhibitor p21, is differentially expressed during growth, differentiation and progression in human melanoma cells. *Oncogene*, **10**, 1855–1864.
- Kannan, P., Buettner, R., Chiao, P.J., Yim, S.O., Sarkiss, M. and Tainsky, M.A. (1994) N-ras oncogene causes AP-2 transcriptional self-interference which leads to transformation. *Genes Dev.*, **8**, 1258–1269.
- Kopf, A.W., Salopek, T.G., Slade, J., Marghoob, A.A. and Bart, R.S. (1995) Techniques of cutaneous examination for the detection of skin cancer. *Cancer*, **75**, 684–690.
- Kozlowski, J.M., Hart, I.R., Fidler, I.J. and Hanna, N. (1984) A human melanoma line heterogeneous with respect to metastatic capacity in athymic nude mice. *J. Natl Cancer Inst.*, **72**, 913–917.
- Lassam, N. and Bickford, S. (1992) Loss of c-KIT expression in cultured melanoma cells. *Oncogene*, **7**, 51–56.
- Leask, A., Byrne, C. and Fuchs, E. (1991) Transcriptional factor AP-2 and its role in epidermal-specific gene expression. *Proc. Natl Acad. Sci. USA*, **88**, 7948–7952.
- Lee, W., Haslinger, A., Karin, M. and Tjian, R. (1987) Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature*, **325**, 368–372.
- Lehmann, J.M., Holzmann, B., Breitbart, E.W., Schmiegelow, P., Riethmüller, G. and Johnson, J.P. (1989) MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecule of the immunoglobulin superfamily. *Proc. Natl Acad. Sci. USA*, **89**, 9891–9895.
- Li, L., Price, J.E., Fan, D., Zhang, R.D., Bucana, C.D. and Fidler, I.J. (1989) Correlation of growth capacity of human tumor cells in hard agarose with their *in vivo* proliferative capacity at specific metastatic sites. *J. Natl Cancer Inst.*, **81**, 1406–1412.
- Luca, M., Huang, S., Gershenwald, J.E., Singh, R.K., Reich, R. and Bar-Eli, M. (1997) Expression of interleukin-8 by human melanoma cells upregulates MMP-2 activity and increases tumor growth and metastasis. *Am. J. Pathol.*, **151**, 1105–1113.
- Luca, M., Hunt, B., Bucana, C.D., Johnson, J.P., Fidler, I.J. and Bar-Eli, M. (1993) Direct correlation between MUC18 expression and metastatic potential of human melanoma cells. *Melanoma Res.*, **3**, 35–41.
- Luca, M., Xie, S., Gutman, M., Huang, S. and Bar-Eli, M. (1995) Abnormalities in the CDKN2 (*p16^{INK4}/mts-1*) gene in human melanoma cells: Relevance to tumor growth and metastasis. *Oncogene*, **11**, 1399–1402.
- Lüscher, B., Mitchell, P.J., Williams, T. and Tjian, R. (1989) Regulation of transcription factor AP-2 by the morphogen retinoic acid and by second messengers. *Genes Dev.*, **3**, 1507–1517.
- Mitchell, P.J., Timmons, P.M., Hebert, J.M., Rigby, P.W. and Tjian, R. (1991) Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. *Genes Dev.*, **5**, 105–119.
- Mitchell, P.J., Wang, C. and Tjian, R. (1987) Positive and negative regulation *in vitro*: Enhancer-binding protein AP-2 is inhibited by SV40 antigen. *Cell*, **50**, 847–861.

- Naeve,G.S., Sharma,A. and Lee,A.S. (1992) Identification of a 10-base pair protein binding site in the promoter of the hamster h3.2 gene required for the S-phase-dependent increase in transcription and its interaction with a jun-like nuclear factor. *Cell Growth Differ.*, **3**, 919–928.
- Natali,P.G., Nicotra,M.R., Digiesi,G., Cavaliere,R., Bigotti,A., Trizio,D. and Segatto,O. (1994) Expression of gp185 HER-2 in human cutaneous melanoma: Implications for experimental immunotherapeutics. *Int. J. Cancer*, **56**, 341–346.
- Natali,P.G., Nicotra,M.R., Winkler,A.B., Cavaliere,R., Bigotti,A. and Ullrich,A. (1992) Progression of human cutaneous melanoma is associated with loss of expression of c-KIT protooncogene receptor. *Int. J. Cancer*, **52**, 197–201.
- Nishikawa,S., Kusakabe,M., Yoshingaga,K., Ogawa,M., Hayashi,S.I., Kunisada,T., Era,T., Sakakura,T. and Nishikawa,S.I. (1991) *In vitro* manipulation of coat color formation by a monoclonal anti-c-KIT antibody: two distinct waves of c-KIT dependency during melanocyte development. *EMBO J.*, **10**, 2111–2118.
- Nocka,K., Majumder,S., Chabot,B., Ray,P., Cervone,M., Bernstein,A. and Besmer,P. (1989) Expression of the c-kit gene products in known cellular targets of *W* mutations in normal and *W* mutant mice: Evidence for impaired c-kit kinase in mutant mice. *Genes Dev.*, **3**, 816–826.
- Nocka,K., Tan,J.C., Chiu,E., Chu,T.Y., Ray,P., Traktman,P. and Besmer,P. (1990) Molecular bases of dominant negative and loss of function mutations at the murine c-KIT/white spotting locus: W^{37} , W^V , W^{41} . *EMBO J.*, **9**, 1805–1813.
- Nyborg,J.K. and Dynan,W.S. (1990) Interaction of cellular proteins with the human T cell leukemia virus type I transcriptional control region. *J. Biol. Chem.*, **265**, 8230–8236.
- Orr-Urtreger,A., Avivi,A., Zimmer,Y., Givol,D., Yarden,Y. and Lonai,P. (1990) Developmental expression of c-kit, a proto-oncogene encoded by the *W* locus. *Development*, **109**, 911–923.
- Pathak,S., Drwinga,H.L. and Hsu,T.C. (1983) Involvement of chromosome 6 in the rearrangement of human malignant melanomas. *Cytogenet. Cell Genet.*, **36**, 573–579.
- Radinsky,R., Fidler,I.J., Price,J.E., Esumi,N., Tsan,R., Petty,C.M., Bucana,C.D. and Bar-Eli,M. (1994) Terminal differentiation and apoptosis in experimental lung metastases of human osteogenic sarcoma cells by wild-type p53. *Oncogene*, **9**, 1877–1883.
- Reith,A.D., Rotapel,R., Giddens,E., Brady,C., Forrester,L. and Bernstein,A. (1990) *W* mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the c-KIT transmembrane receptor. *Genes Dev.*, **4**, 390–400.
- Romerdahl,C.A., Donawho,C., Fidler,I.J. and Kripke,M.L. (1988) Effect of UV-B radiation on the *in vivo* growth of murine melanoma cells. *Cancer Res.*, **48**, 4007–4010.
- Schorle,H., Meier,P., Buchert,M., Jaenisch,R. and Mitchell,P.J. (1996) Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature*, **381**, 235–238.
- Singh,R.K., Gutman,M., Reich,R. and Bar-Eli,M. (1995) Ultraviolet B irradiation promotes tumorigenic and metastatic properties in primary cutaneous melanoma via induction of interleukin 8. *Cancer Res.*, **55**, 3669–3674.
- Tan,J.C., Nocka,K., Ray,P., Traktman,P. and Besmer,P. (1990) The dominant W^{42} spotting phenotype results from a missense mutation in the c-KIT receptor kinase. *Science*, **247**, 209–212.
- Trent,J.M., Stanbridge,E.J., McBride,H.L., Meese,E.U., Casey,G. and Araujo,D.E. (1990) Tumorigenicity in human melanoma cell lines controlled by introduction of human chromosome 6. *Science*, **247**, 569–571.
- Trent,J.M., Thompson,F.H. and Meyskens,F.L. (1983) Identification of a recurring translocation site involving chromosome 6 in human malignant melanoma. *Cancer Res.*, **49**, 420–423.
- van den Oord,J.J., Vandeghinste,N., De Ley,M. and De Wolf-Peters,C. (1994) Bcl-2 expression in human melanocytes and melanocytic tumors. *Am. J. Pathol.*, **145**, 294–300.
- Vidal,M.J., Loganzo,F., Jr, de Oliveira,A.R., Hayward,N.K. and Albino,A.P. (1995) Mutations and defective expression of the WAF-1 p21 tumor-suppressor gene in malignant melanomas. *Melanoma Res.*, **5**, 243–250.
- Walker,G.J., Nancarrow,D.J., Walters,M.K., Palmer,J.M., Weber,J.L. and Hayward,N.K. (1994) Linkage analysis in familial melanoma kindreds to markers on chromosome 6p. *Int. J. Cancer*, **59**, 771–775.
- Welch,D.R., Chen,P., Miele,M.E., McGary,C.T., Bower,J.M. and Weissman,B.E. (1994) Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity. *Oncogene*, **9**, 255–262.
- Williams,T. and Tjian,R. (1991) Characterization of a dimerization motif in AP-2 and its function in heterologous DNA-binding proteins. *Science*, **251**, 1067–1071.
- Williams,T., Admon,A., Luscher,B. and Tjian,R. (1988) Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. *Genes Dev.*, **2**, 1557–1569.
- Xie,S., Huang,S., Luca,M., Gutman,M., Reich,R., Johnson,J.P. and Bar-Eli,M. (1997a) Expression of MCAM/MUC18 by human melanoma cells leads to increased tumor growth and metastasis. *Cancer Res.*, **57**, 2295–2303.
- Xie,S., Price,J.E., Luca,M., Jean,D., Ronai,Z. and Bar-Eli,M. (1997b) Dominant-negative CREB inhibits tumor growth and metastasis of human melanoma cells. *Oncogene*, **15**, 2069–2075.
- Yamamoto,K., Tojo,A., Aoki,N. and Shibuya,A. (1993) Characterization of the promoter region of the human c-KIT protooncogene. *Jpn. J. Cancer Res.*, **84**, 1136–1144.
- Yarden,Y. et al. (1987) A human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J.*, **6**, 3342–3351.
- Zakut,R., Perlis,R., Eliyahu,S., Yarden,Y., Givol,D., Lyman,S.D. and Halaban,R. (1993) KIT ligand (mast cell growth factor) inhibits the growth of KIT-expressing melanoma cells. *Oncogene*, **8**, 2221–2229.
- Zeng,Y.X., Somasundaran,K. and El-Deiry,W.S. (1997) AP-2 inhibits cancer cell growth and activates p21^{WAF1/CIP1} expression. *Nature Genet.*, **15**, 78–82.
- Zhang,J., Cui,P., Glatfelter,A.A., Cummings,L.M., Meltzer,P.S. and Trent,J.M. (1995) Microdissection-based cloning of a translocation breakpoint in a human malignant melanoma. *Cancer Res.*, **55**, 4640–4645.
- Zhang,J., Hagopian-Donaldson,S., Serbedzija,G., Elsemore,J., Plehn-Dujowich,D., McMahon,A.P., Flavell,R.A. and Williams,T. (1996) Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. *Nature*, **381**, 238–241.
- Zsebo,K.M. et al. (1990) Stem cell factor is encoded at the *SL* locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell*, **63**, 213–224.

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