Efficacy of IGFBP7 for treatment of metastatic melanoma and other cancers in mouse models and human cell lines

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Abstract
We recently identified the secreted protein IGFBP7 as a factor required for an activated BRAF oncogene to induce senescence or apoptosis in primary human cells. In human melanomas containing an activating BRAF mutation (BRAF-positive melanomas), IGFBP7 is epigenetically silenced, which seems to be a critical step in melanoma genesis. Restoration of IGFBP7 function by the addition of recombinant IGFBP7 (rIGFBP7) induces apoptosis in BRAF-positive human melanoma cell lines, and systemically administered rIGFBP7 markedly suppresses the growth of BRAF-positive primary tumors in xenografted mice. Here we further evaluate the role of IGFBP7 in the treatment of BRAF-positive melanoma and other malignancies. We find that in human metastatic melanoma samples IGFBP7 is epigenetically silenced and at an even higher frequency than that found in primary melanomas. Using a murine experimental metastasis assay, we show that systemic administration of rIGFBP7 markedly suppresses the growth of metastatic disease and prolongs survival. An analysis of the NCI60 panel of human cancer cell lines reveals that in addition to melanoma, IGFBP7 induces apoptosis in several other cancer types, in particular colorectal cancer cell lines. In general, IGFBP7 induces apoptosis in human cancer cell lines that have an activating mutation in BRAF or RAS, and that are sensitive to chemical inhibition of BRAF-MEK-ERK signaling. Significantly, systemically administered rIGFBP7 blocks the growth of colorectal tumors containing an activating RAS or BRAF mutation in mouse xenografts. The results presented here, in conjunction with those from previous studies, justify the further development of IGFBP7 as an anticancer agent. [Mol Cancer Ther 2009;8(11):3009–14]

Introduction
The proto-oncogene RAF encodes a serine-threonine protein kinase that functions as an immediate downstream effector of RAS (reviewed in ref. 1). RAF activates the MAP kinase extracellular signal-regulated kinase (MEK), which in turn phosphorylates and activates extracellular signal-regulated kinases 1 (ERK1) and 2 (ERK2). Activating mutations in RAF promote cell proliferation and transformation by constitutively activating the RAF-MEK-ERK signaling pathway. Activating BRAF mutations are found at high frequency in human cancers and are particularly prevalent in melanoma, in which they occur at a frequency of 50% to 70% (2).

Paradoxically, when expressed in primary cells, an activated BRAF mutant can block cellular proliferation by inducing senescence or apoptosis (3, 4). Recently, we identified 17 genes required for activated BRAF-mediated apoptosis and senescence, one of which encodes the secreted protein IGFBP7 (4). An analysis of human tissue samples indicates that loss of IGFBP7 expression is a critical step in melanoma development. Most importantly, we found that recombinant IGFBP7 (rIGFBP7) induces apoptosis in BRAF-positive human melanoma cell lines, and systemically administered rIGFBP7 markedly suppresses growth of BRAF-positive melanoma in xenografted mice. Growth suppression results both from inhibition of BRAF-MEK-ERK signaling and activation of an apoptotic pathway that culminates in the upregulation of BNIP3L, a proapoptotic BCL2 family protein.

The selective sensitivity of activated BRAF-containing human cancer cell lines to IGFBP7 and the ability of IGFBP7 to suppress BRAF-positive tumor growth in mouse xenografts suggest a possible role for IGFBP7 in treating BRAF-positive malignancies. Here we further evaluate the potential role of IGFBP7 for treatment of melanoma and other cancers.

Materials and Methods

Immunohistochemistry
The study was approved by the University of Massachusetts Medical Center institutional review board (IRB #12543). Archival materials from metastatic melanoma were retrieved from the pathology files of Boston University School of Medicine, Boston, MA. The histologic sections of all cases were re-reviewed and the diagnoses confirmed by a dermatopathologist (MM). All patient data were de-identified. Immunohistochemical analysis was done as previously described (4). BRAF genotyping was carried out using mutant allele-specific amplification–PCR as previously described (5). The
PCR reaction was done using forward primers 5′-TAGGT-GATTTTGGTCTAGCTACAGT-3′ (to amplify wild-type BRAF) and 5′-GGTGATTTTGGTCTAGCTACAAA-3′ (to amplify the mutant BRAFV600E allele) and reverse primer 5′-GGCCAAAATTTAATCAGTGGA-3′ using the following conditions: denaturation for 2 min at 94°C, followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 45 s at 52°C, and extension for 45 s at 72°C.

Figure 1. Analysis of IGFBP7 expression in human metastatic melanoma samples. 

A, immunohistochemical analysis of IGFBP7 expression in representative human metastatic melanoma tissue samples. As a positive control, IGFBP7 expression is shown in a primary melanoma sample. Samples were stained with H&E. Images are shown at 2× and/or 20×. 

B, summary of IGFBP7 expression in human metastatic melanoma samples. The BRAF status is shown; all samples were negative for the NRASQ161R mutation.

C, bisulfite sequence analysis of the IGFBP7 promoter in human metastatic melanoma tissue samples.
Bisulfite Sequencing

Bisulfite modification was carried out essentially as previously described (4). Six clones were sequenced for each human tissue sample using nested primers BisulBP7-For1 (5′-AGAAGTTTAAATATTGAT-3′), BisulBP7-For2 (5′-GGAAATGGGGAGAAATTAGA-3′) and BisulBP7-Rev2 (5′-GTTGGGTTGTTTTTG TT-3′).

Tumor Formation Assays

Recombinant IGFBP7 (rIGFBP7) was produced and purified from baculovirus-infected cells as previously described.

Figure 2. IGFBP7 suppresses tumor growth and increases survival in an experimental metastasis assay. A, on day 1, rIGFBP7 or PBS was delivered by tail vein injection followed by introduction of A375M-Fluc cells on day 2 (n = 5 mice/group). Two additional doses of rIGFBP7 were administered by tail vein injection on days 3 and 6. Survival probability was calculated using Kaplan-Meier analysis. Right, bioluminescent imaging on day 7. B, on day 1, A375M-Fluc cells were introduced by tail vein injection followed by administration of rIGFBP7 or PBS by tail vein injection on days 5, 8, and 11 (n = 5 mice/group). Right, bioluminescent imaging on day 7.
In the experiments of Fig. 2A, rIGFBP7 (100 μg in 100 μL) or PBS was injected into the tail vein of athymic Balb/c (nu/nu) mice (Taconic; n = 5 mice/group). One day later, the mice were injected through the tail vein with 7 × 10^5 A375M-Fluc cells (a kind gift of Sanjiv Gambhir, Stanford University, in June 2007; ref. 6), and 3 and 6 d later with rIGFBP7 (20 μg) or PBS. On day 7 the mice were injected with D-Luciferin and imaged using a Xenogen IVIS imaging system. Survival probability was calculated using Kaplan-Meier analysis. In the experiments of Fig. 2B, 7 × 10^5 A375M-Fluc cells were injected through the tail vein, and 5, 8, and 11 d later rIGFBP7 (20 μg in 100 μL total volume) was injected (n = 5 mice/group). Animal experiments were done in accordance with the Institutional Animal Care and Use Committee guidelines.

For colorectal cancer cell experiments, 5 × 10^6 HT29 or SW-620 cells were injected s.c. into the right flank of athymic Balb/c (nu/nu) mice (n = 5 mice/group). When tumors reached a size of 100 mm^3, 100 μg rIGFBP7 were delivered by tail vein injection on days 6, 9, and 12. Tumor dimensions were measured every 3 d and tumor volume was calculated using the formula π/6 × (length) × (width)^2.

### Analysis of NCI60 Cell Lines

The NCI60 panel of human cancer cell lines was obtained from the National Cancer Institute (NCI) in September 2006, and grown in RPMI with 10% FCS. Cells were plated and treated with 10 μg rIGFBP7 for 24 h and analyzed for apoptosis induction by Annexin V staining, or treated with U0126 (10 μmol/L; Cell Signaling Technology Inc.) for 24 h and analyzed for proliferation by trypan blue exclusion. All experiments were done in triplicate.

### Results

#### IGFBP7 Is Epigenetically Silenced at High Frequency in Metastatic Melanoma

Our previous study focused exclusively on human primary melanoma samples and mouse models of primary melanoma (4). However, metastatic disease represents the major unmet need for melanoma treatment (7). To evaluate the potential role of IGFBP7 in metastatic melanoma, we examined IGFBP7 expression by immunohistochemistry in a series of human metastatic melanoma samples. Each sample was also analyzed for the presence of the activating BRAFV600E mutation. The results (representative examples are shown in Fig. 1A and the results are summarized in Fig. 1B) reveal that all 20 metastatic melanomas analyzed failed to express detectable levels of IGFBP7, regardless of BRAF status.

To investigate whether the loss of IGFBP7 expression in metastatic melanomas resulted from epigenetic silencing, we did bisulfitesequencing analysis on eight of the samples, which either contained or lacked the activating BRAFV600E mutation. Significantly, all eight samples contained dense hypermethylation of the IGFBP7 promoter (Fig. 1C), indicative of epigenetic silencing and explaining the lack of detectable IGFBP7 expression.

#### IGFBP7 Suppresses Tumor Growth and Increases Survival in a Mouse Model of Metastatic Melanoma

We previously showed that IGFBP7 suppressed tumor growth in a mouse xenograft model of primary melanoma (4). We were therefore interested in determining whether IGFBP7 could also be used to treat metastatic disease, which would be the most important clinical application. For these

![Figure 3](http://example.com/figure3.png)

**Figure 3.** Susceptibility of NCI60 human cancer cell lines to treatment with IGFBP7 or a MEK inhibitor. **A**, cell lines were treated with 10 μg rIGFBP7 for 24 h and analyzed for apoptosis by Annexin V staining. **B**, cell lines were treated with U0126 (10 μmol/L) for 24 h and analyzed for proliferation by trypan blue exclusion. All experiments were done in triplicate. Error bars, SE.
experiments, we used an established murine experimental metastasis assay in which human melanoma cells form pulmonary metastases following tail vein injection (see, for example, refs. 6, 8). The experiments used A375M-Fluc cells, which are a highly metastatic BRAFV600E-positive human melanoma cell line expressing the firefly luciferase (Fluc) gene (6), which enables bioluminescent optical imaging.

In the first set of experiments, in which we asked whether rIGFBP7 could function prophylactically, rIGFBP7 or PBS (as a control) was delivered by tail vein injection on day 1, followed by the introduction of A375-Fluc cells on day 2. Two additional doses of rIGFBP7 were administered by tail vein injection on days 3 and 6. Bioluminescent imaging on day 7 revealed substantial pulmonary metastasis in all untreated animals, whereas pulmonary metastasis was undetectable in animals receiving rIGFBP7 treatment (Fig. 2A). More importantly, all untreated animals died by day 20, whereas rIGFBP7-treated animals survived through day 30, when the experiment was terminated.

In a second set of experiments, A375-Fluc cells were first introduced by tail vein injection (day 1). Subsequently, rIGFBP7 or PBS was administered by tail vein injection on days 3, 5, and 11. Bioluminescent imaging on day 7, prior to IGFBP7 administration, revealed that measurable pulmonary tumors were present prior to initiation of therapy (Supplementary Fig. S1). On day 7, substantial pulmonary metastasis was observed in all untreated animals, whereas pulmonary metastasis was undetectable in all animals receiving rIGFBP7 (Fig. 2B). None of the untreated animals survived beyond day 24, whereas all the animals receiving rIGFBP7 treatment survived through day 50, when the experiment was terminated.

**Susceptibility of NCI60 Human Cancer Cell Lines to IGFBP7 Treatment**

Activating BRAF mutations are also found in a number of other solid tumors, including colorectal, ovarian, and non-small cell lung cancers (2). In addition, up to 30% of solid tumors contain activating RAS mutations, which can also increase BRAF-MEK-ERK signaling (reviewed in ref. 9). We therefore investigated the potential use of IGFBP7 in the treatment of other cancers. Toward this goal, we analyzed the NCI60 panel of human cancer cell lines for sensitivity to apoptosis induced by rIGFBP7. In parallel, we tested the ability of the chemical MEK inhibitor, U0126, to block proliferation of the NCI60 cell lines. The BRAF or RAS mutational status in each of the NCI60 cell lines was derived from previously published data (10). The results, shown in Fig. 3, enable us to draw several conclusions.

First, consistent with our previous findings (4), we found that human melanoma cell lines were highly sensitive to IGFBP7-mediated apoptosis. Second, the vast majority of the NCI60 cell lines were unaffected by rIGFBP7, as expected for a targeted therapeutic as opposed to a general cytotoxic agent. Third, several breast, ovarian, lung, and, in particular, colorectal cancer cell lines underwent significant apoptosis following the addition of rIGFBP7. Finally, in general the human cancer lines that were sensitive to IGFBP7-mediated apoptosis contained an activating BRAF or RAS mutation and were also sensitive to U0126-mediated growth arrest. We note, however, that there were several exceptions to this general trend. For example, several breast cancer (BT-549 and MCF7) and colorectal cancer (HCC-2998 and KM-12) cell lines lacked an activating BRAF or RAS mutation and were sensitive to IGFBP7 but not to U0126. Conversely, several human cancer cell lines such as HS 578T (breast), A549 (lung), and CCRF-CEM (hematopoietic) contained activating RAS mutations and were sensitive to U0126 but not to IGFBP7.

**IGFBP7 Suppresses Tumor Growth in Human Colorectal Cancer Mouse Xenografts**

The ability of rIGFBP7 to induce apoptosis in human colorectal cancer cell lines raised the possibility that rIGFBP7 could suppress the growth of colorectal tumors. To test this possibility we chose two human colorectal cancer cell lines, one of which contained an activating BRAF mutation (HT29) and the second of which contained an activating KRAS mutation (SW-620). Each cell line was injected s.c. into the flanks of nude mice and when tumors reached a size of 100 mm³, 100 μg rIGFBP7 or PBS was delivered by tail vein injection on days 6, 9, and 12. Figure 4 shows that systemic administration of rIGFBP7 completely suppressed growth of both HT29 and SW-620 tumors.

**Discussion**

Here we conducted a series of experiments to further investigate the potential role of IGFBP7 in the treatment of melanoma and other cancers. Of particular interest was metastatic melanoma, an aggressive disease that is refractory to conventional chemotherapeutic agents and lacks adequate treatment options (reviewed in ref. 7). Similar to our previous results using a primary melanoma model (4), we found that systemically administered rIGFBP7 suppressed tumor growth and prolonged survival in a murine experimental metastasis assay. Analysis of IGFBP7 in the
recently developed mouse models of BRAF-positive melanoma (11, 12) remains an important future objective.

We had previously shown that IGFBP7 expression is lost in primary melanomas bearing an activating BRAF mutation but not in primary melanomas with wild-type BRAF (4). However, here we found that IGFBP7 expression was undetectable in all metastatic melanomas analyzed, regardless of BRAF status. The higher rate of loss of IGFBP7 expression in metastatic samples suggests that during melanoma development there is a strong selection against IGFBP7 expression, providing further evidence that IGFBP7 is a melanoma tumor suppressor gene.

An analysis of the NCI60 panel of cell lines revealed that IGFBP7 induced apoptosis in several cancer types in addition to melanoma. In general, IGFBP7 induced apoptosis in human cancer cell lines that had an activating mutation in BRAF or RAS, and that were sensitive to chemical inhibition of BRAF-MEK-ERK signaling. Previous studies have shown that cancer cells harboring an activated BRAF mutation are highly dependent on BRAF-MEK-ERK signaling for proliferation and survival (13), and that BRAF mutation predicts sensitivity to MEK inhibition (14). These findings provide the rationale for developing therapeutic strategies that target the BRAF-MEK-ERK signaling pathway for treatment of melanoma and other cancers in which BRAF is mutated (13). Inhibitors of BRAF have been developed but unfortunately have done poorly in clinical trials (15, 16).

We have previously shown that IGFBP7 inhibits BRAF-MEK-ERK signaling and efficiently induces apoptosis in BRAF-positive melanoma cell lines (4). The ability of IGFBP7 to both inhibit BRAF-MEK-ERK signaling and irreversibly induce apoptosis following transient exposure may make it particularly efficacious for treating malignancies that are dependent upon BRAF-MEK-ERK signaling.

We found that colorectal cancer cell lines were also highly susceptible to IGFBP7-mediated apoptosis, consistent with the high frequency of activating BRAF or RAS mutations, and presumed increase BRAF-MEK-ERK signaling, in colorectal cancers (1, 9). Significantly, previous studies have found that IGFBP7 expression is lost in human colorectal cancers (17, 18), consistent with the possibility that IGFBP7 is a colorectal cancer tumor suppressor (19, 20). Most importantly, we found that systemically administered rIGFBP7 markedly suppressed growth of colorectal tumors containing an activating RAS or BRAF mutation in mouse xenografts. These collective results support the encouraging possibility that IGFBP7 may also have a role in the treatment of colorectal cancer.

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

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