Up-regulation of MET Expression by α-Melanocyte-stimulating Hormone and MITF Allows Hepatocyte Growth Factor to Protect Melanocytes and Melanoma Cells from Apoptosis*

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The MET proto-oncogene encodes for the hepatocyte growth factor (HGF) receptor, a plasma membrane tyrosine kinase that is involved in melanocyte growth and melanoma development. In mouse melanoma cells, Met expression is increased by αMSH via the activation of the cAMP pathway. However, the mechanism by which cAMP regulates MET and the biological consequences of this increase were not known. In the present report, we show that αMSH regulates MET expression in both human melanocytes and mouse melanoma cells through a transcriptional mechanism that requires MITF. Furthermore, the adeno-virus driven expression of MITF is sufficient to increase MET in melanoma cells. Functional analysis of the MET promoter allows us to identify an E-box motif conserved in both human and mouse promoter that mediates the effect of MITF. Interestingly, up-regulation of MET expression by cAMP leads to an exacerbated HGF signaling and allows HGF to protect melanocytes and melanoma cells from apoptosis. Thus, physiological stimuli or pathological events that would induce MITF expression may lead to increased MET expression thereby favoring melanoma survival. These observations strengthen the roles of MITF and MET in melanoma development.

The MET proto-oncogene encodes for the hepatocyte growth factor receptor, a plasma membrane tyrosine kinase involved in the pleiotropic physiological effects and tumorigenic processes of numerous tissues.

Upon HGF binding, the intracellular part of b-subunit undergoes autophosphorylation on tyrosine residues creating a unique docking site for PI3K3 or GRB2-SOS adaptors that allow the activation of ERK signaling pathways. The C-terminal part of MET also interacts with the non-receptor tyrosine kinase Src, the transcription factor STAT3, and the adapters Shc and Gab1 (1). All of these partner molecules allow MET to activate numerous signaling pathways and induce various physiological effects, including cell motility, cell growth, tubulogenesis, B-cell differentiation, and muscle and nervous system development (2).

The role of MET in growth and motility, often called “invasive growth” places MET at a center stage of tumor and metastasis development. Indeed, MET overexpression is very common in human cancer. Gain-of-function mutations in the tyrosine kinase domain of the receptor have been identified in hereditary papillary renal carcinoma type I and in other sporadic tumors (3).

HGF/MET signaling has is. Indeed, in transgenic mice over expressing HGF, melanosis in central nervous system and skin hyperpigmentation were observed (4). HGF can replace steel factor, the Kit ligand, to promote melanoblast growth and differentiation from neural crest explants (5).

MET is expressed on melanocytes and HGF is a growth factor for cultured melanocytes (6). HGF/MET has been shown to influence melanocyte transformation and the acquisition of the metastatic phenotype (7). Furthermore, HGF appears to play a key role in the regulation of melanoma interaction with its microenvironment, since HGF controls E-cadherin (8), CD44v6 (9), fibronectin (10), as well as MMP2 (11) expression in melanoma cells.

It is noteworthy that transgenic mice overexpressing HGF in their skin develop melanoma upon UV radiation of the neocytes thereby recapitulating the etiologic role of UV in human melanoma genesis (12).

Recently, overexpression of MET in human melanoma samples has been correlated with a poor clinical outcome (13).

HGF and MET play a key protective role against apoptosis (14). Indeed, HGF counters anoikis-induced apoptosis (15) that is particularly relevant when cancer cells enter the bloodstream to form distant metastasis. Interestingly, MET also favors cell survival, in a HGF-independent process, by interacting and sequestering FAS (16). In melanocytes or in melanoma cells, the role of HGF/MET in apoptosis has not been studied so far, although we have shown that HGF does not protect human melanocytes from Trail induced apoptosis (17).

Induction of melanocyte differentiation by αMSH and other cAMP-elevating agents, such as forskolin, has been extensively

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3 The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; HGF, hepatocyte growth factor; αMSH, α-melanocyte-stimulating hormone; PARP, poly(ADP-ribose) polymerase; RT, reverse transcription; siRNA, small interfering RNA; CAT, chloramphenicol acetyltransferase.

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Adenoviral Infection and siRNA—Adenovirus encoding wild-type Mitf (Ad-Mitf) was described previously (21). siRNA to mouse or human MITF were described previously (22, 23). Vector Constructions and Mutagenesis—pCDNA3 vectors encoding wild-type MITF (Mitf) has previously been described (24). The 1.6-kb mouse promoter fused to Cat reporter gene (pMet) was kindly provided by Seol (25). ΔpMet was constructed by digesting pMet by SplI and KpnI. E-box mutations were performed using the QuickChange site-directed mutagenesis kit (Stratagene) and the following primers: 5′-GAGCGGAGAACAGATCCGGGTTGGGAC-3′ (for pMetM1), 5′-GTTCGGGCCTCAGGCTGGTGAACCCGAGCGGCTC-3′ (for pMetM2), and 5′-GTGACCTGTGCGGACGTCCGATGCCTGGCG-ACCGCGTCTC-3′ (for pMetM3). An 800-bp fragment of the human MET promoter (AF046925) was cloned using the following primers: 5′-CGACATCCACTTACCTCCGCTC-3′ (forward). Reverse-5′-CCCTCCCCGCTCTCCTCAC-3′.

Nuclear Extracts and Gel Mobility Shift Assay—Nuclear extracts from control cells or cells incubated with forskolin for 5 h were prepared as described previously (26). The Met promoter specific probe spanning the E-box2 (E2), 5′-GTTCGGGCCCAGGAG-3′ (for pMETmE1), 5′-GTGGTACTGACGTGCTGCGG-3′ (for pMETmE2), and the mutant probe (mut E2), 5′-GTTCGGGCCCAGGAGTCCTGGATTGCCTGCGG-3′, were γ-32P-end-labeled using T4 polynucleotide kinase. 5 μg of nuclear proteins were preci-

bined in a binding buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 4% glycerol, 80 μg/ml salmon sperm DNA, 0.1 μg of poly(dI-dC), 10% fetal calf serum, 2 mM MgCl2, and 2 mM spermidine for 15 min on ice. Then, 50,000 cpm of 32P-labeled probe were added to the binding reaction for 10 min at room temperature. DNA–protein complexes were resolved by electrophoresis on a 4% polyacrylamide gel (37:5:1 acrylamide–bisacrylamide) in TBE buffer (22.5 mM Tris borate, 0.5 mM EDTA, pH 8) for 1.5 h at 100 V. For supershift assays, 0.5 μl of preimmune serum or anti-MITF antibody (26) was preincubated with nuclear extracts in the binding reaction buffer before adding the labeled probe.

Western Blot Assays—The cells were grown in 6-well dishes and treated as indicated in the figure legends, then solubilized in buffer A containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 μM leupeptin, 1 mM (2-aminomethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 100 units/ml aprotinin, 10 mM NaF, and 1 mM Na3VO4. The samples (30 μg) were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride, and then exposed to the appropriate antibodies. The proteins were visualized with the ECL system (Amersham Biosciences).

Transfection, Chloramphenicol Acetyltransferase (CAT), and Luciferase Assays—B16 melanoma cells were seeded in 6-well dishes, and 24 h later, the cells were co-transfected with 2 μg of promoter/CAT reporter construct, 0.2 μg of β-galactosidase expression vector, and 1 μg of empty pCDNA3 or pCDNA3-Mitf expression vector. The transfection was performed using 10 μl of Lipofectamine reagent (Invitrogen) in 800 μl of Opti-MEM for 6 h. The transfection medium was then replaced with fresh medium and 48 h later the cells were extracted with reporter lysis buffer (Promega).
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![Image 1](https://example.com/image1)

**FIGURE 1.** cAMP pathway increases MET expression in melanocytes and melanoma cells. A, mouse B16 melanoma cells; B, normal human melanocytes (NHM); C, human melanoma cells, MEL501, were treated with 0.1 μM HGF, 1 μM forskolin (Fsk), or 1 μM αMSH for 8 h. The proteins were analyzed by Western blot using monoclonal antibodies against MET and MITF. ERK2 antibody was used as a loading control.

The CAT assay was performed according manufacturer’s instructions. The β-galactosidase plasmid was co-transfected to monitor transfection efficiency and the CAT activity was corrected by the activity of β-galactosidase in each experiment. The luciferase assays were performed as described previously (24).

**RESULTS**

αMSH Increases MET Expression in Both Human Melanocytes and Mouse Melanoma Cells—In B16F10 melanoma cells and in human melanocytes, we showed that αMSH and forskolin treatments, for 8 h, increased MET and MITF levels (Fig. 1, A and B). MET expression that reached a maximum after 8 h of forskolin was slightly delayed compared with the MITF expression profile that peaked at 5 h (data not shown). Conversely, treatment of B16 melanoma cells with 0.1 μM HGF for 8 h led to a decrease in Met and Mitf expression (Fig. 1A). HGF has no significant effect on MITF and MET expression in human melanocytes (Fig. 1B).

In human MEL501 melanoma cells, HGF also decreased MET expression but did not significantly affect MITF level (Fig. 1C). Forskolin or αMSH did not increase MITF or MET expression. The cAMP pathway does not seem to be functional in these cells.

Real-time RT-PCR analysis showed a clear increase in Met messengers, 4 h after forskolin addition. The maximum stimulation was reached at 8 h and remained stable, at least until 24 h (Fig. 2A). Furthermore, cAMP-elevating agents, forskolin and αMSH, increased (4–6-fold) Met promoter activity in CAT reporter assay (Fig. 2B). Interestingly, HGF, which decreased Met expression at the protein level, slightly activated Met promoter.

This set of observations indicates that cAMP increases MET expression through a transcriptional regulation. The correlation between MET and MITF up-regulation upon activation of the cAMP pathway suggests that MITF might be involved in this process.

![Image 2](https://example.com/image2)

**FIGURE 2.** cAMP pathway increases MET expression at the transcriptional level. A, the relative amount of Met mRNA in B16 melanoma cells treated with 1 μM forskolin over the indicated time was determined by quantitative real-time RT-PCR. All results were normalized to 36B4 values. The results are the mean ± S.D. from three independent experiments. B, B16 cells were transfected with pMet reporter plasmid and then treated with HGF (50 ng/ml) or forskolin (1 μM). Met promoter activity was determined using a classical CAT assay. The results are the mean ± S.D. from three independent experiments.

MITF Mediates the Effect of cAMP on MET and Is Sufficient to Regulate MET Expression—To verify this hypothesis we used specific siRNA to silence Mitf (20). Real-time RT-PCR analysis showed that Mitf messengers were dramatically decreased, in both basal and forskolin-stimulated conditions, after transfection with siMitf compared with scramble siRNA (si-SC). In each case, all transcript levels were normalized to 36B4 values. The results are the mean ± S.D. from three independent experiments. B, Western blot analysis of Met, tyrosinase (Tyr), Mitf, and total Erk2 in B16 treated as described for A. C, Western blot analysis of MET, MITF, and ERK2 in MEL501 transfected with either siRNA to Mitf (si-Mitf) or scrambled siRNA (si-SC).

![Image 3](https://example.com/image3)

**FIGURE 3.** MITF is required for MET expression in melanocytes and melanoma cells. A, real-time RT-PCR assays of Mitf, Tyrosinase, and Met mRNA levels in untreated or forskolin-treated B16 melanoma cells transfected with either siRNA to Mitf (si-Mitf) or scrambled siRNA (si-SC). In each case, all transcript levels were normalized to 36B4 values. The results are the mean ± S.D. from three independent experiments. B, Western blot analysis of Met, tyrosinase (Tyr), Mitf, and total Erk2 in B16 treated as described for A. C, Western blot analysis of MET, MITF, and ERK2 in MEL501 transfected with either siRNA to Mitf (si-Mitf) or scrambled siRNA (si-SC).
expression and, consequently, the effect of cAMP on tyrosinase and Met expression (Fig. 3B). Interestingly, the basal levels of Met in B16 melanoma cells as well as in human melanoma, Mel501 (Fig. 3C) were dramatically reduced by MITF silencing, indicating that basal and cAMP-stimulated Met expression, in melanoma cells, required the presence of MITF. However, we still had to determine whether MITF was sufficient to up-regulate Met expression. For this purpose, human A375 melanoma cells, expressing a very low level of MITF, were infected with mouse Mitf encoding adenovirus. The levels of mouse and human MITF, mouse Mitf, TYR, MET, and HIF1α transcripts in A375 human melanoma cells infected with an adenovirus encoding wild-type mouse Mitf (ad-Mitf). All results were normalized to samples infected with control adenovirus and 5834 values. The results are the mean ± S.D. of three independent experiments. B and C: A375 melanoma cells (B) or human normal melanocytes (C) infected with an adenovirus encoding mouse Mitf or an empty adenovirus were analyzed by Western blot using monoclonal antibodies against MET and MITF. ERK2 antibody was used as a loading control.

**FIGURE 4. MITF is sufficient for MET expression in melanocytes and melanoma cells.** A, real-time RT-PCR analysis was used to determine the levels of the human MITF, mouse Mitf, TYR, MET, and HIF1α transcripts in A375 human melanoma cells infected with an adenovirus encoding wild-type mouse Mitf (ad-Mitf). All results were normalized to samples infected with control adenovirus and 5834 values. The results are the mean ± S.D. of three independent experiments. B and C: A375 melanoma cells (B) or human normal melanocytes (C) infected with an adenovirus encoding mouse Mitf or an empty adenovirus were analyzed by Western blot using monoclonal antibodies against MET and MITF. ERK2 antibody was used as a loading control.

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MITF Stimulates the MET Promoter Activity through an E-box Motif Conserved in Both Mouse and in Human Sequences—Next, we performed a functional analysis of the Met promoter to identify the Mitf binding site. We used the 1.6-kb mouse Met promoter fused to CAT reporter gene (pMet) described by Seol et al. (25). This sequence shows a strong homology with the human MET promoter. The regions in black in Fig. 5. A display over 70% identity, and the proximal region contains 3 E-boxes that could bind Mitf (Fig. 5A). Transfection of a Mitf expression vector led to an 8-fold stimulation of the promoter activity (Fig. 5B). First, we deleted 1.2 kb from the 5’ end to remove E-box1. The new construct ΔpMet contains 400 bp of the Met promoter including 2 E-boxes. The response of this construct to Mitf was identical to that of the full-length promoter, indicating that Mitf responses were mediated by cis-acting elements found in this proximal fragment. We analyzed the effect of the individual mutation of the three E-boxes (−13/−19, −83/−89, and −234/−240) contained in this fragment. As expected from the results obtained with the deletion construct, mutation of E-box1 (−234/−240) did not affect Mitf effects. Similarly, mutation of E-box3 (−13/−19) did not impair Mitf effects. However, mutation of the E-box2 (−83/−89) dramatically reduced the activation of the Met promoter by Mitf. The same experiments using the human MET promoter upstream the luciferase reporter gene (Fig. 5C) confirmed that the MET promoter is stimulated by Mitf through the E-box2 that is perfectly conserved in both human and mouse promoters.

Gel shift assay using a probe encompassing the E-box 2 showed an increased binding of nuclear proteins in extract from forskolin treated cells (Fig. 5D). This binding was displaced by an excess of cold E-box2 but not by the mutated E-box2. Furthermore, Mitf was identified as the nuclear protein bound to E-box2, since the E-box2 complex was shifted by antibody to Mitf.

HGF Signaling and Anti-apoptotic Effects Are Exacerbated in Melanocytes and Melanoma Cells Exposed to Forskolin—Analysis of the HGF signaling pathway in B16 melanoma cells (Fig. 6A) showed that in basal conditions, the addition of HGF led to a slight, yet significant activation of Akt and Erk, monitored by Western blot analysis with phospho-Akt and phospho-Erk antibodies. After 8 h of exposure to forskolin, Met and Mitf expression markedly increased. In these conditions, the addition of HGF induced a stronger phosphorylation, and thus activation of Akt compared with basal conditions. Interestingly, after forskolin treatment, HGF led to a huge Erk phosphorylation. This high Erk activity is reflected by the Mitf shift from a doublet to a single upper band, corresponding to Erk phosphorylated Mitf on serine 73 (28). Western blots with anti-Erk2 antibody were performed to ensure the even loading of each lane.

Thus cAMP elevating agents increase Met expression and exacerbate Met signaling.

Finally, we investigated the biological consequences of the increased Met expression and signaling. We studied the anti-apoptotic effect of HGF in normal human melanocytes and in B16 melanoma cells exposed or not to forskolin. An evaluation of
melanocyte viability showed that TRAIL induced death in normal human melanocytes (Fig. 6B). HGF was not able to protect normal human melanocytes from TRAIL-induced death. When the cells were first exposed to forskolin for 15 h, before treatment with TRAIL, we observed a slight increase in cell viability. In these cells, HGF completely blocked cell death induced by TRAIL.

We also analyzed two classical apoptosis parameters, caspase 3 and PARP cleavage. Consistent with the survival data, in control B16 melanoma cells, staurosporine treatment led to a decrease in caspase 3 expression that reflected its cleavage and activation (Fig. 6C). As a consequence, we observed a cleavage of PARP monitored by an antibody specific for the cleaved form of PARP. In these cells HGF neither prevented caspase 3 nor PARP cleavage. Conversely, in cells pretreated with forskolin, to up-regulate Met, HGF markedly decreased the effect of staurosporine on caspase 3 activation and PARP cleavage.

Taken together, these observations indicate that the up-regulation of MET by cAMP and MITF allows HGF to protect melanocytes and melanoma cells from apoptosis.

DISCUSSION

In the present report, we demonstrated that cAMP elevating agents, αMSH and forskolin, increase MET expression in melanocyte and melanoma cells through a transcriptional mechanism that requires MITF. Furthermore, MITF overexpression is sufficient to increase HIF1A, BCL2, and MET levels but not tyrosinase expression. The tyrosinase gene, as well as the genes encoding other melanogenic proteins, appears to have a non-permissive chromatin conformation. Indeed, MITF needs to recruit the SWI/SNF enzymes that allow chromatin remodelling and melanogenic gene transcription (29). HIF1A, BCL2, and MET, which do not require SWI/SNF enzymes, appear to be in an active genomic region. This suggests that overexpression of MITF in pathological conditions such as genomic amplification (30) would be sufficient to induce MET overexpression. However, one should not conclude that MET expression is controlled exclusively by MITF. Indeed, analysis of several melanoma cell lines did not show a tight correlation between MET and MITF expression. However, in melanoma cell lines
expressing both MITF and MET, MITF silencing dramatically reduced MET expression. In melanoma cells, regulation of MET expression probably depends on ubiquitous transcription factors such as SP1 (31) or AP1 (32) that control MET expression in other cell systems. More recently, hypoxia, through HIF1A, has been shown to regulate MET expression in osteosarcoma cells (33). Since we have shown that cAMP, through MITF, regulates HIF1A expression in melanoma, it was tempting to propose that HIF1A mediates the effect of MITF on MET. However, in non-hypoxic conditions, HIF1A silencing did not affect MET expression in melanoma cells (data not shown) suggesting that MITF acts directly on MET. This hypothesis is strengthened by studies on the MET promoter. Analysis of 5’ non-coding sequence of the mouse Met promoter showed a strong homology with human MET gene. In this region 3 E-boxes are conserved in both human and mouse sequences. Functional analysis of the mouse promoter showed a strong stimulation of its activity in melanoma by MIF that is mainly mediated by the E-box in position −83/−89 that binds MIF. This E-box is conserved in the human MET gene and also mediates MITF responsiveness of the human MET promoter.

Our data, identifying MITF as the transcription factor that mediates the effects of cAMP on MET expression in melanocytes and melanoma cells, agree with the recent paper by McGill (34) reporting that MITF mediated the up-regulation of MET by its own ligand, HGF. However, important differences should be highlighted between our data and that of the report mentioned above. In our hands, it has never been possible to show an increase in MET protein expression induced by HGF. On the contrary, we consistently observe an inhibition of MET at the protein level, with a slight increase in the MET messengers. Our observations are consistent with numerous reports demonstrating that HGF increases MET transcription through activation of the AP1 transcription factor (32). However, at the same time HGF decreases MET protein level through a classical desensitization mechanism shared by membrane tyrosine kinase receptors (35). Therefore, the results obtained by McGill (34) do not seem to be consistent with the literature and might be explained by the use of an anti-Met antibody that recognizes RON, a closely related tyrosine kinase receptor, that is indeed up-regulated by HGF (36).

On the basis of a gel shift assay, McGill et al. (34) have identified E-box at position −300 in the human MET promoter as the sequence that binds MITF. According to a previous report from Comoglio’s group (33), this E-box is located at +19/
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ERK and PI3K pathways (14). Thus, the robust activation of these two pathways by HGF in melanocytes and melanoma cells exposed to forskolin might account for the anti-apoptotic effect of HGF in these cells (Fig. 7).

Interestingly, the tyrosine phosphatase SHP2 (PTPN11) is required for MET signaling to ERK and PI3K pathways and mutations in SHP2 lead to generalized lentigines found in the LEOPARD syndrome. These observations support the role of MET in melanocyte biology.

The role of HGF/MET in the survival of melanoma cells has not been extensively studied and as far as we know this the first report demonstrating that HGF/MET can protect melanoma cells from apoptosis. This effect of the HGF/MET cascade might be particularly important for the pro survival effects provided by HGF derived from the stromal environment of the tumor (40). Furthermore, at the beginning of the invasive process, melanoma cells enter the bloodstream to disseminate and form distant metastasis. Up-regulation of MET could favor melanoma survival in anoikis conditions and allow transient growth in an anchorage-independent manner.

These effects of MET are not necessarily dependent on HGF, since MET interacts with other membrane receptors with adhesive functions such as CD44 and integrin (41). MET can also block apoptosis through its direct interaction with Fas (16) and melanoma cells are particularly resistant to Fas ligand-induced apoptosis. Since melanoma cells are more sensitive to TRAIL, it would be interesting to investigate the possible interaction of MET with TRAIL receptors (DR4, DR5).

The up-regulation of MET by the activation of the cAMP pathway is particularly relevant in the case of solar irradiation. Indeed, UV radiations are known to increase the production of αMSH and stimulate the synthesis of pro-inflammatory agents such as prostaglandins. Both αMSH and prostaglandins through G-protein coupled receptor activate the cAMP and could stimulate MET expression as a response to solar exposure or acute inflammation.

Taken together the data presented in this report shows that the up-regulation of MITF expression by cAMP or by other physiological or pathological stimuli would lead to increased expression of MET thereby favoring melanoma survival. These observations strengthen the roles of MITF and MET in melanoma development.

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