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Hepatocyte Growth Factor/Scatter Factor and Hepatocytes are Potent Downregulators of Tyrosinase Expression in B16 Melanoma Cells

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Abstract Reiterated selection in vivo of B16 murine melanoma cells for enhanced liver metastatic ability yielded a cell line (B16-LS9) dramatically overexpressing a constitutively active hepatocyte growth factor/scatter factor (HGF/SF) receptor, the product of the c-met proto-oncogene. Most likely because of their overexpressing c-met, B16-LS9 cells appear to be more responsive than parental B16-F1 cells to HGF stimulation, in terms of motility, invasion, and growth. They are also more pigmented, and express higher levels of tyrosinase as compared to parental B16-F1 cells. Therefore, we set out to explore whether HGF/SF and the liver might influence the differentiation state of B16 cells. We found that HGF/SF and MSH, two factors which reportedly have a strong influence on the phenotype and the malignant behavior of melanoma cells, may act at different levels, and with opposite results, on the regulation of gene expression. In fact, while MSH induces, at the transcriptional level, an increase in the production of both c-met and tyrosinase, HGF/SF, in contrast, promotes a decrease in the expression of both c-met and tyrosinase, however at a posttranscriptional level. These two opposite effects can counter-balance each other, when the cells are treated with both factors at the same time, apparently through a mechanism involving MAP kinase activation. The effects were, however, additive when morphological changes were considered. Most intriguingly, we also describe a very strong downregulatory activity, limited to tyrosinase expression, by hepatocytes in coculture with B16 cells. This activity, also at the posttranscriptional level, is much stronger than that exerted by HGF/SF, and appears to be due to a labile soluble factor produced by the hepatocytes. J. Cell. Biochem. 71:264–276, 1998.

Key words: HGF/SF; MSH; c-met; tyrosinase; B16 melanoma

Mammalian pigmentation results from melanin biosynthesis in melanocytes. The enzymatic reactions responsible for pigment formation occur in specialized organelles—melanosomes—starting with the conversion of tyrosine in dihydroxyphenylalanine (DOPA) and DOPAquinone, which are dependent on the enzyme tyrosinase [Körner and Pawelek, 1982]. Therefore, without enzymatic action of tyrosinase, the melanin synthetic pathway is blocked. Tyrosinase gene expression is tightly controlled in time and space, and in the adult organism it is restricted to melanocytes [Beermann et al., 1992; Schedl et al., 1993]. Tyrosinase expression is usually regulated at the transcriptional level, depending on both positive and negative regulatory elements working in concert to modulate promoter activity. Cell type specific expression seems to be achieved by specific promoter repression, while positive promoter elements rather modulate the expression levels [Ganss et al., 1994]. However, melanocyte-specific transcription factors have not yet been identified, and only indirect evidence links the microphthalmia gene product to the regulation of the tyrosinase promoter by cAMP [Bertolotto et al., 1996].

B16 murine melanoma cells have been and still are widely used as an in vivo/in vitro model system to address specific questions related to the differentiated phenotype, or the metastatic behavior of malignant tumor cells. In our laboratory we have selected and characterized B16 cell lines with enhanced liver metastatic capabilities [Tao et al., 1980; Sargent et al., 1988; Rusciano et al., 1993]. Liver-specific B16-LS9 cells preferentially colonize the liver of syn-

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genic animals mainly because of paracrine growth effects depending on their interaction with hepatocytes [Rusciano et al., 1993]. Next, we have shown that growth of B16 cells as liver colonies leads to an enhanced expression of the proto-oncogene c-met, the receptor for hepatocyte growth factor/scatter factor [Rusciano et al., 1995; Lin et al, 1998]. As a consequence of its over-expression, c-met is constitutively activated in B16-LS9 cells [Rusciano et al., 1996], which then become highly responsive to stimulation by exogenous HGF/SF [Rusciano et al., 1995]. B16-LS9 cells appear to be more differentiated than parental B16-F1 cells, in that they are more pigmented, and express higher amounts of tyrosinase. Since treatment with melanocyte stimulating hormone (MSH), a potent inducer of differentiation in melanocytes and melanomas, upregulates both tyrosinase and c-met expression in B16 melanoma cells at a transcriptional level [Rusciano et al., unpublished observations], we address here the question whether HGF/SF treatment may also influence the differentiation state of these cells. We report that HGF/SF antagonizes the effects of MSH, though at the posttranscriptional level, triggering a decrease of both c-met and tyrosinase expression, through a mechanism which might involve MAP kinase (ERK) activation. Moreover, we also report that hepatocytes, only when in coculture with B16 melanoma cells, are potent downregulators of tyrosinase (but not c-met) expression. This effect seems to be mediated by some specific labile factor(s), different from HGF/SF, produced by hepatocytes in presence of B16 cells.

MATERIALS AND METHODS

Cell Culture and Treatment

B16 melanoma cells were routinely cultured as monolayers in Petri dishes (Falcon, Lincoln Park, NJ) in a humidified incubator at 37°C with DMEM 10% FCS, and periodically checked for mycoplasma contamination by DAPI staining. For either subculturing or plating an experiment, cells were detached by EDTA treatment (5 min at 37°C), and the desired number of cells seeded in appropriate plates or dishes (Falcon), as indicated in figure legends. Eight to 18 h after plating, when cells were well adhered on the dish, they were treated with either murine recombinant HGF/SF, kindly provided by E. Gherardi (Cambridge, UK), or with MSH (Sigma, St. Louis, MO), or a combination of the two (see figure legends for details of treatment). Primary mouse hepatocytes were prepared from livers of 50- to 60-days-old C57-Bl/6 male mice by collagenase perfusion, as described [Rusciano et al., 1993], and either plated on the bottom well (no direct cellular contact) or the lower face of a transwell chamber with 3 μm pores (direct cellular contact) in six transwell multiwell plates (Falcon). B16 melanoma cells were plated on the upper surface of the transwell chamber. Hepatocyte conditioned medium was produced either from hepatocytes alone, or in coculture with B16 melanoma cells. 3×10^5 mouse primary hepatocytes/well were plated in six multiwell plates, with or without 3×10^4 B16-LS9 cells, in DMEM with 5% FCS. Medium was replaced with fresh medium every other day for 6 days. Conditioned medium used in the experiment came from the last 48 h of production, and was added, after filtration through 0.22 mm filters (Millex, Millipore Corp., Bedford, MA), to B16 cells plated at 3×10^4/well into six multiwell plates. After 4 days of growth, extracts were prepared in CHAPS buffer, and analyzed by Western immunoblot.

Extracts and Western Immunoblotting

Cell monolayers were rinsed 3× with cold PBS, and extracted with CHAPS buffer [Rusciano et al., 1995] for 20 min at 4°C. The extraction temperature is critical, because we have shown that the amount of c-met tyrosine phosphorylation strongly depends on a balance between tyrosine kinase and phosphatase activities, which favors either one at different temperatures [Rusciano et al., 1996]. Proteins in the extracts were determined by the Bio-Rad Dc protein determination kit, and equal amounts of proteins were first resolved on a 6% SDS-PAGE under reducing conditions, and next blotted to a PVDF membrane (Immobilon, Millipore Corp.). Equal loading was controlled by amido-black staining of the membrane, which was then destained by 30 min incubation at room temperature in saturation buffer (Tris/HCl 100 mM pH 7.4, $MgCl_2$ 100 mM, Tween-20 0.5%, Triton-X-100 1%, BSA 1%, FCS 5%). Antibodies were diluted in the same saturation buffer, and detection of secondary peroxidase-labeled antibodies was done by luminol (ECL, Amersham, Buckinghamshire, UK) on Hyperfilm MP (Amersham). Anti-phosphotyrosine mouse monoclonal antibody 4G10 was a kind gift of Kurt Ballmer (Friedrich Miescher Insti-

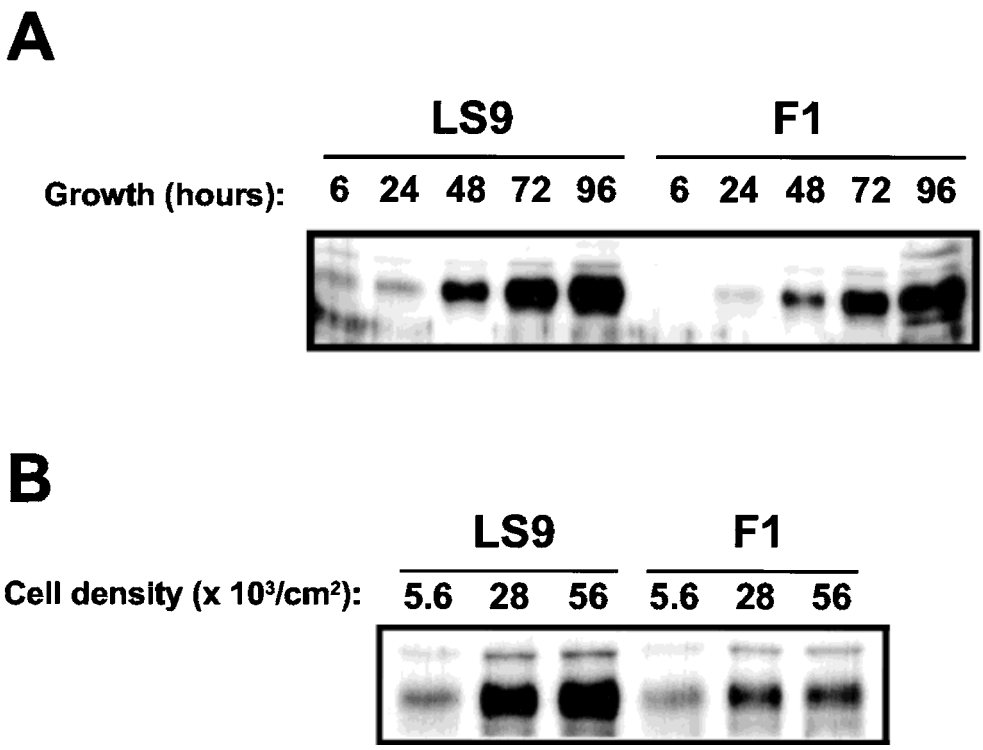


Fig. 1. Tyrosinase expression levels in B16 melanoma cells. **A:** 5×10^4 B16 cells (LS9 or F1) were plated in 35 mm Petri dishes, with 5% FCS-containing DMEM. At different times, as indicated, total cell extracts were made in CHAPS buffer (see Materials and Methods), and 30 µg of protein per lane analyzed by western immnunoblotting with anti-tyrosinase (anti-pep7) antibodies. **B:** B16 cells were plated as above, but at different densities, as indicated. Total cell extracts in CHAPS buffer were prepared and analyzed as above 24 h later.

tut); anti-active-MAPK (ERK-1 and -2) polyclonal antibody was from New England Biolabs (Beverly, MA); polyclonal antibodies against MAPK (ERK-1 and -2) and anti-mouse c-met were from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal antiserum against mouse tyrosinase (anti-pep7) was kindly donated by V.J. Hearing (NIH, Bethesda, MD).

Melanin determination was done by optical reading at 490 nm after extraction of the cell monolayer with hot NaOH 1 M/10 % DMSO.

RNA Extraction and Northern Blotting

B16 cells were plated at the desired concentration (see figure legends) in 10 cm dishes, with 5% FCS. The next day HGF/SF (70 ng/ml), or MSH (10 mM) were added to the different dishes. At different times, as indicated in figure legends, mRNA was prepared with the aid of the mRNA Quick Prep (micro) kit (Pharmacia Biotech, Gaithersburg, MD), and 3mg of mRNA blotted to a positively charged nylon membrane (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Equal loading of the lanes was estimated after methylene blue staining of the membrane. A 2.2 kB c-met probe was kindly provided by Walter Birchmeier (Berlin, Germany); mouse tyrosinase full length coding sequence was obtained from ATCC. Antisense RNA probes were labeled with digoxigenin by the DIG RNA labeling kit (Boehringer-Mannheim Biochemicals). Alkaline phosphatase on anti-digoxigenin labeled antibodies was developed with the chemiluminescent substrate CSPD (Tropix, Bedford, MA).

RESULTS

Differentiation State of B16 Cells

Liver-specific B16-LS9 cells were derived from parental B16-F1 cells after nine repeated passages as hepatic metastatic colonies in the liver of syngeneic C57Bl/6 male mice [Rusciano et al., 1993]. In comparison to B16-F1, B16-LS9 showed a decreased growth rate both in vitro and in vivo [Rusciano et al., 1993], and increased melanin content (Fig. 3B), which are both hallmarks of melanoma differentiation. Figure 1 clearly illustrates that the higher mela-

nin content of B16-LS9 cells correlates with a higher amount of tyrosinase expression. In agreement with previous reports [Wade and Burkart, 1978; Jiang et al., 1995], we found that the amount of tyrosinase expression increased during logarithmic growth of the cells (Fig. 1A), most likely because of an increase in the cell density (Fig. 1B). In both cases, however, B16-LS9 cells were found to express more tyrosinase than parental B16-F1. B16-LS9 cells overexpress the HGF/SF receptor (c-met), which thus becomes constitutively activated [Rusciano et al., 1996], however leaving the cells highly responsive to exogenous HGF/SF stimulation [Rusciano et al., 1995]. Therefore, we decided to investigate whether there was any relationship between c-met activation and differentiation in B16 melanoma cells.

HGF/SF and MSH Have Opposite Effects on c-met and Tyrosinase Expression

Tyrosinase is the key enzyme in melanin biosynthesis [Hearing and Tsukamoto, 1991], and a hallmark of differentiation in melanocytes and melanoma cells. Therefore, in order to characterize the differentiative effects (if any) of HGF/SF treatment on B16 melanoma cells, we evaluated the influence of such treatment on tyrosinase expression. In comparison, we also treated B16 cells with the differentiative agent MSH. Results of a Western immunoblotting assay are reported in Figure 2A and show that HGF/SF down regulated both c-met and tyrosinase expression. The effects on tyrosinase down regulation were dramatically more pronounced in B16-LS9 than in B16-F1 cells, most likely because of c-met overexpression in the former cell line. MSH, on the contrary, enhanced the expression of both c-met and tyrosinase in the two cell lines. Cell pigmentation changed accordingly, and a parallel decrease in melanin content was observed in cells treated with HGF/SF, while a sharp increase was detected after MSH treatment (Fig. 2B). However, MSH treatment enhanced c-met and tyrosinase expression both at the protein and the mRNA level (Figs. 2A and 3A) [Aroca et al., 1993], whereas the decrease in tyrosinase and c-met expression at the protein level after HGF/SF treatment were not paralleled by a similar decrease at the mRNA level (Fig. 3A,C). In agreement with previous reports [Boccaccio et al., 1994; de Juan et al., 1994], we also observed that in B16-F1 cells HGF/SF treatment initially increased the amount of c-met mRNA (Fig. 3B).

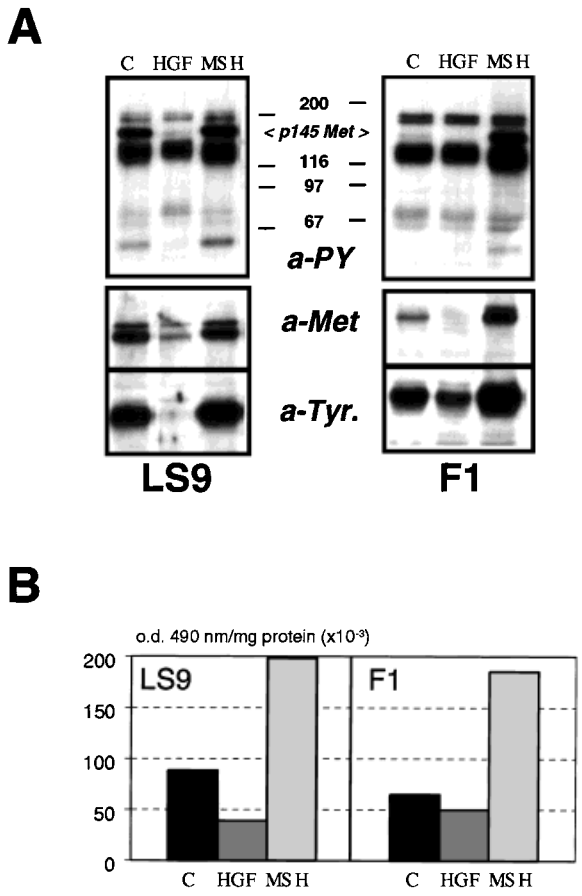


Fig. 2. **A:** Opposite effects of HGF/SF and MSH on c-met and tyrosinase expression. B16 cells were plated at 5×10^4 per well, in six multiwell plates with DMEM and 5% FCS. HGF/SF (70 ng/ml) or MSH (1 mM) were added 16 h after plating, and the culture continued for 48 h, after which time total cell extracts were prepared in CHAPS buffer, and 40 μg of proteins analyzed by Western immunoblot with anti-phosphotyrosine (a-PY), anti-c-met (a-Met), and anti-tyrosinase (a-Tyr) antibodies. **B:** Pigmentation of B16 cells treated with HGF/SF or MSH. B16 cells, plated and treated as above, were extracted with hot 1 M NaOH/10% DMSO, and the solubilized melanin quantitated by optical reading at 490 nm. To take into account the different growth rate of the two cell lines under the different conditions, o.d. was normalized to protein content.

Given thus the opposite effects of HGF/SF and MSH on the expression of both c-met and tyrosinase, at least at the protein level, we set out to test whether either factor was predominant over the other. B16 melanoma cells were thus treated with a combination of the two factors, and c-met activation (tyrosine phosphorylation) and expression, together with tyrosinase expression, were evaluated by western immunoblotting. Figure 4A shows that c-met

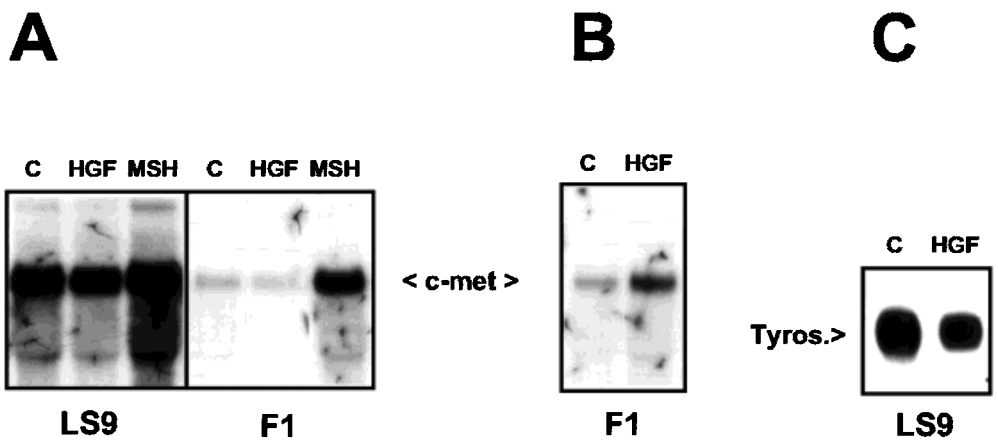
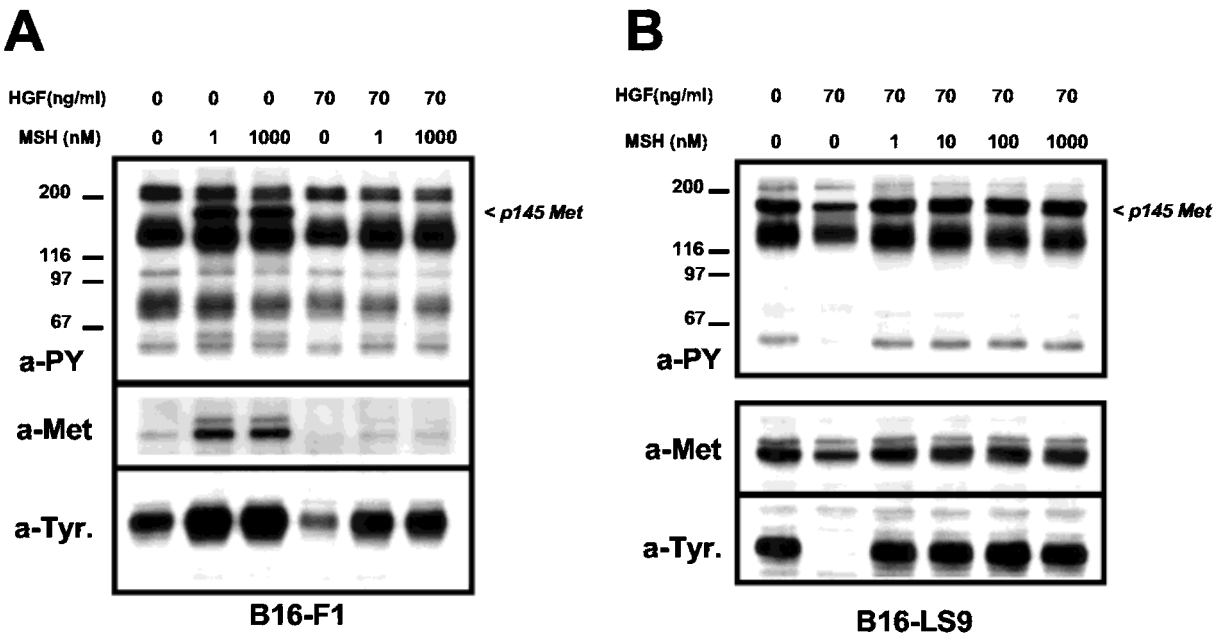


Fig. 3. Northern blot analysis of HGF/SF and MSH effects on c-met and tyrosinase expression. **A,B:** B16 cells were plated at 10^6 per dish in 10 cm petri dishes with DMEM and 5% FCS, and treated for either 48 h (A), or 6 h (B) with HGF/SF (70 ng/ml) or MSH (10 nM). At the end of each time point mRNA was prepared from cell lysates (see Materials and Methods), and 3 µg analyzed by northern blot with a full length probe labeled with digoxigenin. C-met at 7.4 kB is indicated by arrowheads. **C:** 5×10^5 B16-LS9 cells were plated as above, and treated with 70 ng/ml of HGF/SF for 96 h. 3 µg of mRNA from treated and untreated control cells were analyzed by northern blot with a full length, digoxigenin-labeled probe. Tyrosinase at 2.0 kB is indicated by the arrowhead.

induction by MSH in B16-F1 cells was prevented in the presence of HGF/SF. However, tyrosinase and c-met downregulation by HGF/SF were also competed by the simultaneous presence of MSH. Similarly, in B16-LS9 cells (Fig. 4B) MSH, already at 1 nM, was able to block the downregulatory action of HGF/SF on both c-met and tyrosinase. Interestingly, at the morphological level MSH and HGF/SF had additive effects, as in the presence of both factors together B16-LS9 cells were both scattered and presented a more pronounced dendritic phenotype (Fig. 4C).

HGF/SF is known to trigger MAP kinase (mainly ERK-2) activation in melanocytes [Halaban et al., 1992; Böhm et al., 1995], whereas cAMP elevation has been shown to block MAP kinase activation induced by HGF/SF in hepatocytes [Adachi et al., 1996]. We thus tested the effects of HGF/SF on MAP kinase phosphorylation in B16 melanoma cells, under different conditions known to affect cAMP levels. Figure 5A shows the response of B16 cells to HGF/SF. In B16-F1 cells a transient activation (phosphorylation) of ERK-2 was visible at 5 and 15 min after stimulation. In contrast, a prolonged activation was evident in B16-LS9 cells, lasting at least until 3 h after treatment. The phosphorylation response of c-met (lower panels) was similar in both B16-F1 and -LS9 cells, and concordant with the peak of MAP kinase activation. It is interesting to note that B16-LS9 cells, despite a constitutive high level of c-met activation and tyrosine phosphorylation, were further responsive to HGF/SF treatment. MSH treatment, as expected [Englaro et al., 1995] elevated MAP kinase activation, whereas forskolin (a cAMP elevating agent) alone did not (Fig. 5B). MAP kinase activation by HGF/SF was decreased in presence of either forskolin or MSH, although with a different timing, most likely dependent on the different kinetics on cAMP elevation of the two agents: in fact, in the presence of forskolin (a direct activator of adenyl-cyclase) the effect was stronger after 5 min of pretreatment, while with MSH (which has to act via receptor activation) a comparable effect was observed after 1 h of pretreatment.

HGF/SF treatment exerted long-lasting effects on c-met and tyrosinase expression in B16-LS9 cells (Fig. 6). After 96 h of treatment, the expected decrease in c-met and tyrosinase expression was observed, with the lower dose of HGF/SF (7 ng/ml) being less effective than the usual dose of 70 ng/ml. After removal of HGF/SF at 48 h, and a further incubation period of 48 h with fresh medium in the absence of HGF/SF, a further decrease of c-met and tyrosinase was observed in the wells treated with 70 ng/ml, and, for tyrosinase only, also in the wells receiving the lower dose of 7 ng/ml.



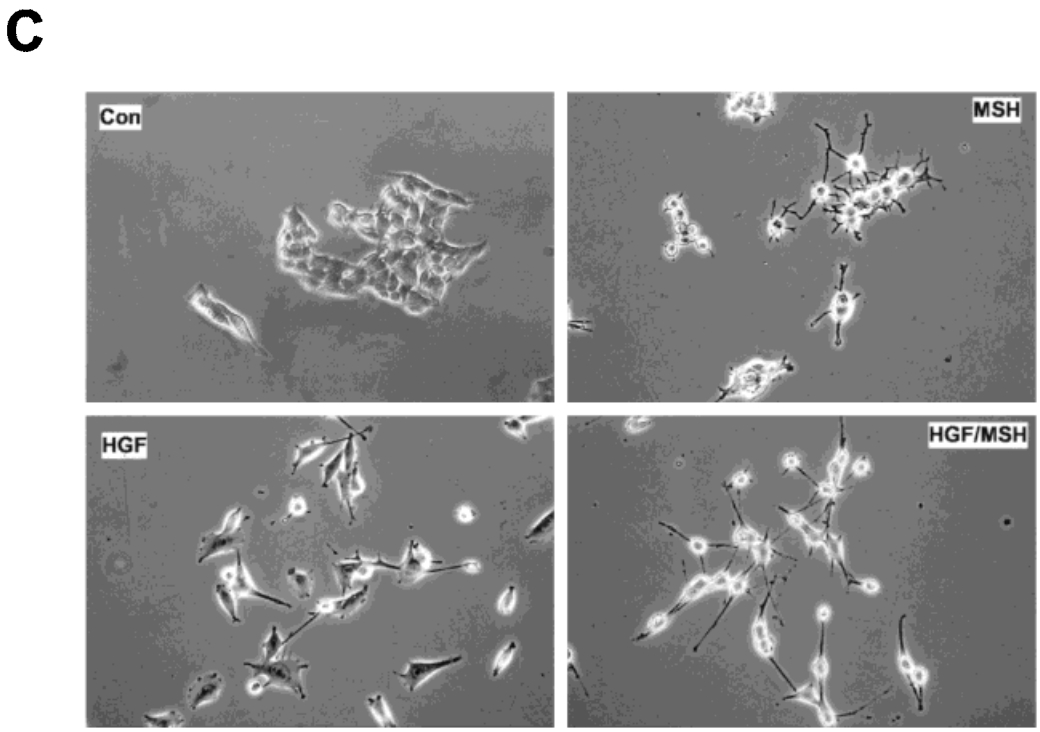
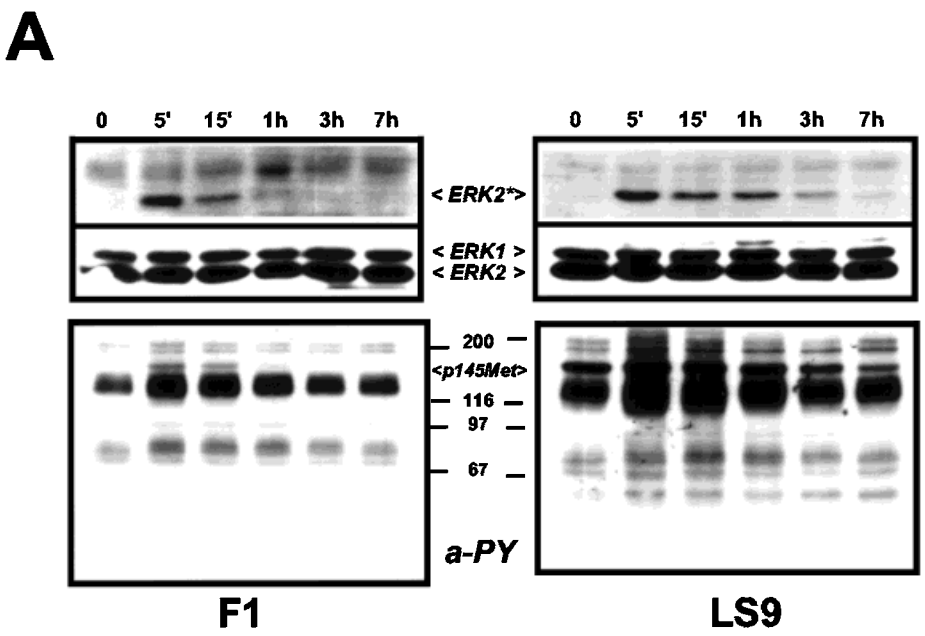


Fig. 4. Antagonistic effects of HGF/SF and MSH. 5×10^4 B16 cells, either F1 (**A**), or LS9 (**B**) were plated per each well of a six multiwell plate in DMEM, 5% FCS, and treated for 48 h with the indicated combinations of HGF/SF and MSH. Total cell extracts were prepared in CHAPS buffer and analyzed by Western immunoblotting with anti-phosphotyrosine (a-PY), anti-c-met (a-Met), and anti-tyrosinase (a-Tyr). **C:** B16 cells were plated at 10^4 per well in a six multiwell plate with DMEM/5 % FCS, and treated with HGF/SF at 70 ng/ml, MSH 1mM, or a combination of the two (as indicated), for 2 days, at which time phase-contrast pictures were taken by a videocamera connected to an inverted microscope, with a 10× objective.



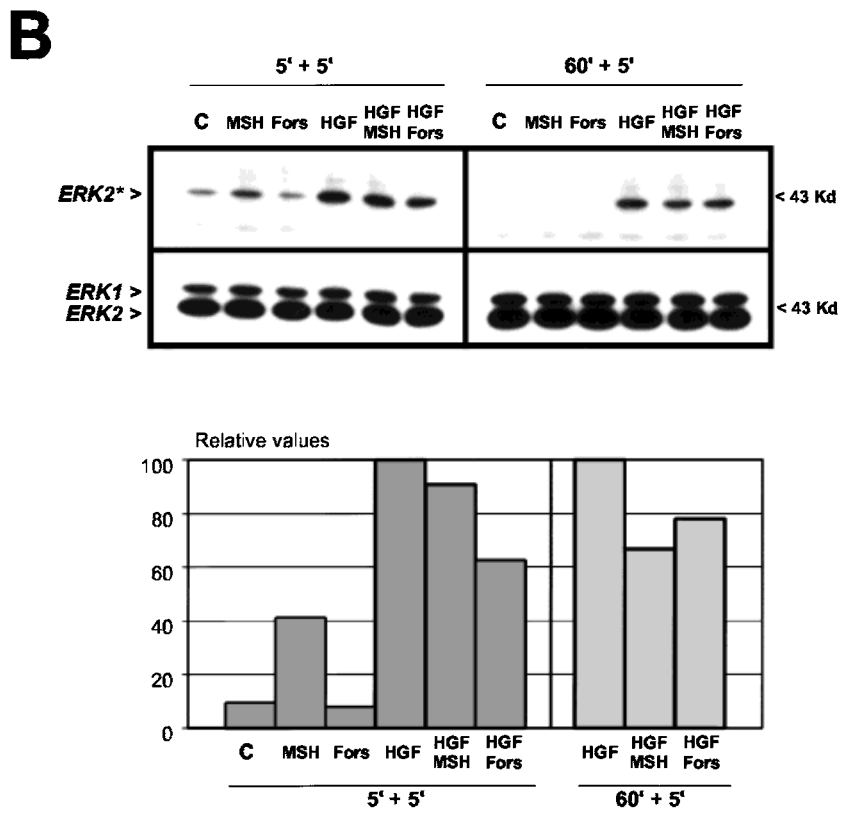


Fig. 5. MAP kinase activation in B16 cells. **A:** 5×10^5 cells/well were seeded in six multiwell plates, in DMEM/5% FCS, and treated with recombinant HGF/SF at 70 ng/ml. At the indicated times, CHAPS extracts were prepared, and 40 mg of proteins analyzed by Western immunoblotting with antibodies specific for either the phosphorylated or the generic form of MAP kinases (ERK-1 and -2: upper panels), and for tyrosine phosphorylated proteins (a-PY: lower panels). **B:** B16-LS9 cells were plated as above, and given a pretreatment with cAMP elevating agents (MSH or forskolin) for either 5 (left panel), or 60 min (right panel), before a 5 min stimulation with HGF/SF. CHAPS extracts were prepared and 30 mg of proteins analyzed as above. Quantitative analysis of phospho-MAP kinase bands is shown at the bottom: values in each experiment have been normalized to the band obtained after HGF/SF induction.

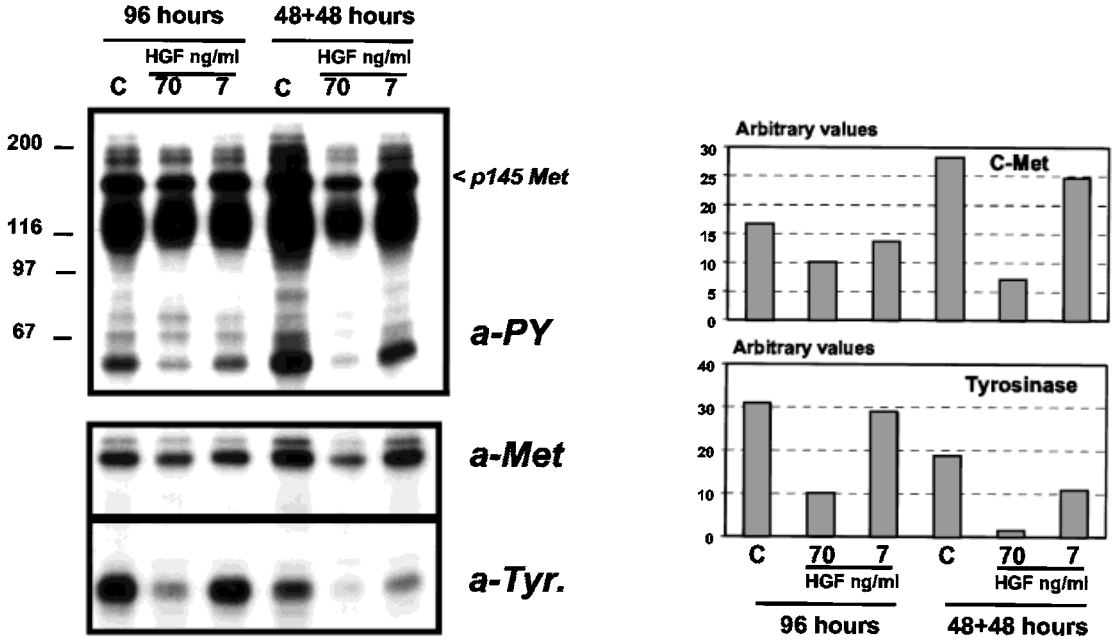
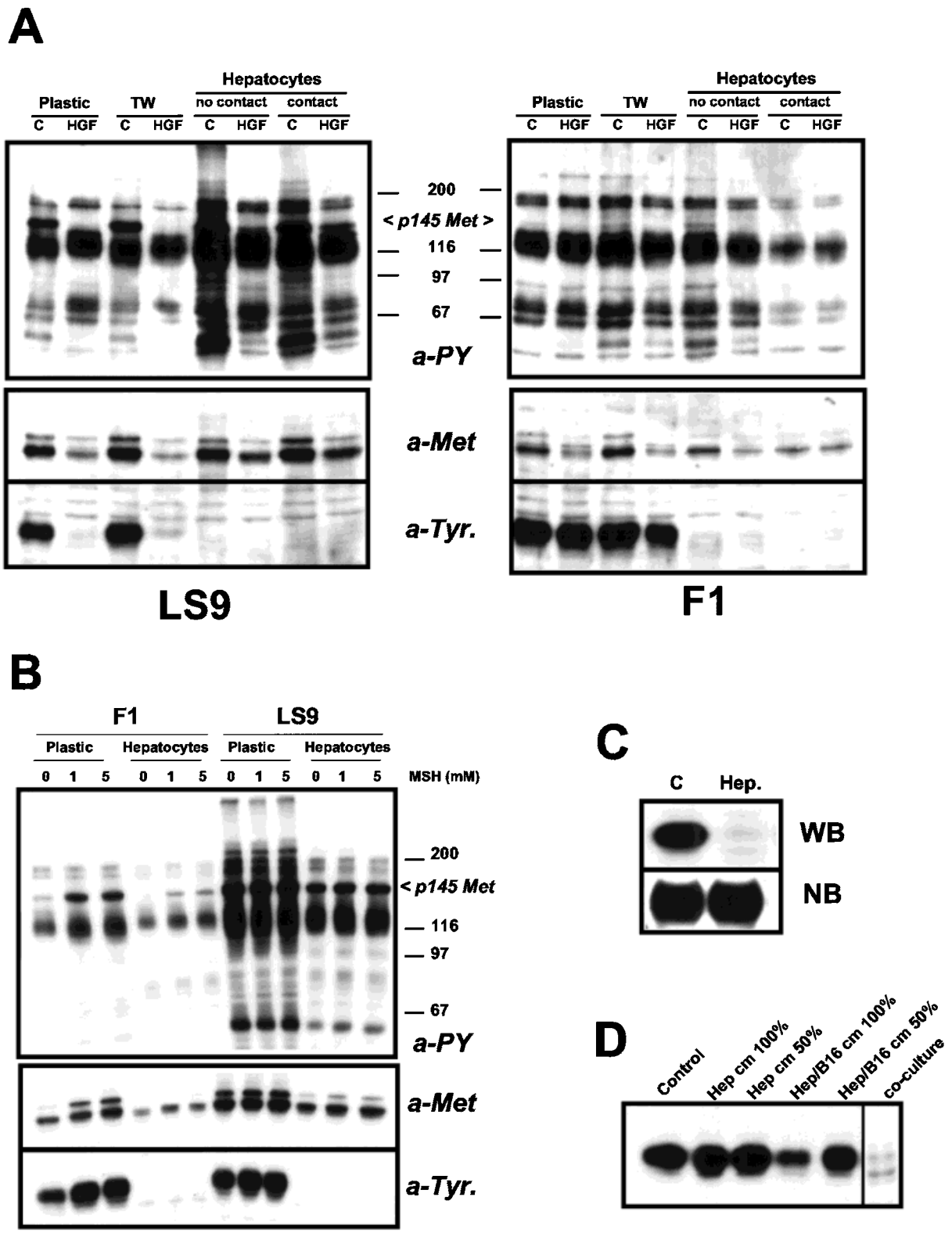


Fig. 6. Reversibility of HGF/SF effects. 10^4 B16-LS9 cells were plated per each well of a six multiwell plate, in DMEM/5 % FCS, and treated with HGF/SF at either 70 or 7 ng/ml, as indicated. Forty-eight hours later three wells (one control and two treated) were rinsed four times with PBS, and fresh culture medium without HGF/SF added, and incubation continued for additional 48 h. At the end of the experiment, total cell extracts were prepared in CHAPS buffer, and 40 µg of proteins analyzed by Western immunoblot with anti-phosphotyrosine (a-PY), anti-c-met (a-Met), and anti-tyrosinase (a-Tyr). The amount of c-met and tyrosinase expression was also quantitated by film scanning, and the arbitrary values thus obtained reported in bar graphs.

Hepatocytes are Potent Downregulators of Tyrosinase Expression

B16-LS9 cells efficiently metastasize to the liver of syngeneic mice because of specific paracrine growth effects at that site, most likely mediated through a direct contact with hepatocytes [Sargent et al., 1988; Rusciano et al., 1993]. The liver is also a producer and a reservoir of HGF/SF [Tashiro et al., 1990; Masumoto et al., 1991]. Therefore, we decided to investigate whether the presence of primary mouse hepatocytes in coculture (either with or without direct contact) would influence the response of B16 melanoma cells to exogenous HGF/SF. Results reported in Figure 7A show that HGF/SF had the expected effect of decreasing both c-met and tyrosinase expression in B16-LS9 and -F1 cells, either when they were plated on plastic or when they were seeded on the transwell porous membrane. The effects on tyrosinase expression were dramatically more evident in B16-LS9 than in B16-F1. Predictably, the presence of hepatocytes in coculture had some growth stimulatory effect, more on B16-LS9 than on B16-F1 cells, which resulted in a higher cell density, and therefore an increase in c-met expression and activation (tyrosine-phosphorylation) [Rusciano et al., 1996]. Treatment with HGF/SF in the presence of hepatocytes resulted in a lesser decrease of c-met expression in B16-LS9 cells, which was more evident when direct contact was achieved. Intriguingly, direct contact between hepatocytes and B16-F1 cells resulted in some downregulation of c-met expression in the latter, which was, however, not further decreased by the addition of HGF/SF. Surprisingly, hepatocytes in coculture had very strong down regulatory effects on tyrosinase expression, both in B16-LS9 and -F1 cells. Most notably, tyrosinase expression in B16-F1 cells, which was only scarcely decreased by HGF/SF, was completely blocked by hepatocytes, either in presence or absence of direct cellular contact. This downregulatory effect was maximal when hepatocytes were simultaneously present in coculture. Medium conditioned by hepatocytes alone had no effect, while a weak effect was visible on tyrosinase (but not c-met) expression when B16-LS9 cells were grown in medium conditioned by hepatocytes and B16-LS9 cells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co-culture

Figure 7.

in coculture (Fig. 7D). Despite a dramatic decrease of tyrosinase at the protein level under co-culture conditions, the amount of the relative RNA message remained stable (Fig. 7C), indicating that, like in the case of HGF/SF, transcriptional regulation could not account for the observed variation in protein expression. However, differently from what observed with HGF/SF, the hepatocyte effect was not competed by MSH (Fig. 7B): tyrosinase was still strongly downregulated by hepatocytes, both in B16-F1 and -LS9 cells, also in the presence of 1 or 5 mM MSH, and c-met was still induced by MSH, also in the presence of hepatocytes, although to a lesser extent.

DISCUSSION

In this paper we show a potent downregulatory effect of HGF/SF and of a soluble, as yet unidentified, hepatocyte factor on tyrosinase expression in B16 melanoma cells.

HGF/SF is known to have morphogenetic activity, inducing branching of kidney [Santos et al., 1994] and breast [Soriano et al., 1995] epithelial cells, and promoting angiogenesis by endothelial cells [Bussolino et al., 1992; Grant et al., 1993]. On melanocytes, HGF/SF had mitogenic effects, and stimulated motility, invasion, tyrosinase activity, and pigmentation [Halaban et al., 1993]. Some of these effects appear to be reversed in melanoma cells, since HGF/SF treatment rather resulted in growth inhibition [Mizuno and Nakamura, 1993; Rusciano et al., 1998]. In this report, we show that—despite the fact that B16-LS9 melanoma cells, overexpressing a constitutively active c-met, presented some features of differentiated cells, such as increased tyrosinase and melanin content (Figs. 1 and 2B), and decreased growth rate [Rusciano et al., 1993]—further stimulation of c-met by HGF/SF treatment resulted in a strong decrease of tyrosinase expression and cell pigmentation (Fig. 2). Also c-met expression was decreased after prolonged HGF/SF treatment of B16 cells, while it was enhanced upon MSH treatment (Fig. 2A). The downregulatory effect of HGF/SF is persistent, and even 48 h after removal of the ligand, a strong decrease in c-met and tyrosinase expression could be observed (Fig. 6). However, despite a transient upregulation of c-met mRNA at 6 h in B16-F1 cells (Fig. 3B), Northern blot analysis did not show a proportional decrease in mRNA levels of either c-met (Fig. 3A) or tyrosinase (Fig. 3C). Therefore, posttranscriptional events should be responsible for the observed decrease in protein expression. Recently, it has been reported that HGF/SF may trigger c-met ubiquitination and degradation in a carcinoma cell line [Jeffers et al., 1997], and it is thus conceivable that a similar mechanism might also lead to the observed decrease of c-met and tyrosinase in B16 cells. However, the kinetics of c-met downregulation by HGF/SF reported by Jeffers and collaborators [1997] appeared to be relatively fast (2 h for a strong decrease of protein levels), and independent of cell density, while in the case of B16 cells the effects were dependent on cell density, and at least 18 h were required to observe a consistent decrease of c-met or tyrosinase expression (not shown). Moreover, when we tried to treat B16 cells with HGF/SF in presence of lactacystin, a

Fig. 7. Hepatocyte effects on c-met and tyrosinase expression. **A:** Mouse primary hepatocytes were plated at 3×10^5 per well either in the bottom well of a six multiwell plate (no contact), or on the lower surface of a 3 mm transwell (TW) membrane (contact), in DMEM with 1% FCS and insulin. The next day, after a thorough rinsing of the hepatocyte culture with PBS, 3×10^3 B16 melanoma cells were plated on the upper surface of the transwell chambers in DMEM 5% FCS, or on plastic as control, and the coculture carried out for 7 days. At this time total cell extracts of B16 cells were prepared in CHAPS buffer, and analyzed by western immunoblot with anti-phosphotyrosine (a-PY), anti-c-met (a-Met), and anti-tyrosinase (a-Tyr). Control extracts (C) prepared from cells grown in the absence of hepatocytes either on plastic or on transwell membrane are also reported for comparison. **B:** 5×10^4 B16 cells were plated either on plastic, or in coculture with 3×10^5 primary mouse hepatocytes, and few hours later, when cells were adherent, different amounts of MSH were added. After 2 to 4 days, when B16 cells were close to confluence, CHAPS extracts were prepared and analyzed by western immunoblotting for tyrosine phosphorylation (a-PY: upper panel), c-met and tyrosinase expression (lower panel). **C:** Western and Northern blot analysis of B16-LS9 cells in transwell coculture with hepatocytes in the absence of cellular contact. 3×10^4 melanoma cells were grown in coculture with hepatocytes for 4 days, at the end of which time some wells were used for Western blot analysis, and duplicate companion wells were taken for mRNA preparation. Forty mg of proteins and 3 mg of mRNA were probed respectively with anti-tyrosinase antibodies (upper panel), or with a digoxigenin-labeled full length tyrosinase probe (lower panel). **D:** Western immunoblot analysis of B16-LS9 cells grown with hepatocyte conditioned medium. B16 cells were plated at 3×10^4 per well in six multiwell plates and treated with different amounts of medium conditioned by hepatocytes either alone (Hep cm) or in coculture (Hep/B16 cm). After 4 days of growth under these conditions, total B16-LS9 cell extracts were prepared and analyzed for c-met and tyrosinase expression. Controls were made from cells grown in regular culture medium, and from cells grown in coculture with hepatocytes.

known inhibitor of the proteasome pathway [Fenteany et al., 1995], we found only minor effects on c-met, and no rescue of tyrosinase expression (not shown), suggesting that a proteasome-mediated degradation is not triggered by HGF/SF in B16 cells. MSH treatment, on the contrary, resulted in a consistent up regulation of both c-met and tyrosinase expression, both at the protein (Fig. 2A) and the mRNA (Fig. 3A) [Englaro et al., 1995] levels. However, neither factor (MSH or HGF/SF) predominated on the other one (Fig. 4A,B). MSH stimulation is known to lead to an elevation of cAMP, which then activates PKA, MAP kinase, and AP1 (JunD/Fra2), thus resulting in an increased transcription of tyrosinase [Englaro et al., 1995]. However, while Englaro and collaborators describe activation of ERK-1, based on the specificity of immunoprecipitating antibodies, we found, consistently with previous reports [Halaban et al., 1992; Böhm et al., 1995; Adachi et al., 1996], activation of ERK-2. cAMP elevation, however, has been shown to block MAP kinase (ERK-2) activation induced in hepatocytes by HGF/SF [Adachi et al., 1996], and we also found a weaker activation of MAP kinase by HGF/SF in presence of cAMP elevating agents (Fig. 5B). It might thus be speculated that the decreased expression of c-met and tyrosinase induced by HGF/SF requires ERK-2 activation, and is thus inhibited by an elevation of cAMP, which reduces the extent of such activation. In keeping with this mechanism, Englaro and coworkers [1998] recently reported that inhibition of the ERK pathway triggered B16 melanoma cell differentiation, while a sustained activation could impair the melanogenic effect of cAMP elevating agents.

Induction of cell motility and morphological differentiation (dendritic phenotype) respectively by HGF/SF and MSH, are not influenced by the simultaneous presence of either factor (Fig. 4C), suggesting that they are under the control of effectors not influenced by cAMP or MAP kinases. A likely candidate is PI 3-kinase, which has been shown to act in concert with the GTPases Rac and Rho to induce reorganization of the actin cytoskeleton [Reif et al., 1996], and to be essential for HGF/SF-induced scattering of MDCK cells [Royal and Park, 1995]. A dramatic suppression of the pigmented phenotype has also been observed in mouse melanocytes transfected with bFGF [Dotto et al., 1989], whereas transfection of human HGF/SF did not lead to a decreased pigmentation [Halaban et al., 1992]. These latter results might, however, depend either on a different response of melanocytes with respect to melanoma cells, or on the reported presence of cAMP elevating agents in the melanocyte culture medium, which may interfere with the effects of HGF/SF (Fig. 5B) [Adachi et al., 1996].

Intriguing and unexpected was the strong downregulatory effect by hepatocytes on tyrosinase expression by B16 cells. Once more, the effect was evident at the protein, but not at the mRNA level, suggesting that hepatocytes produce some labile soluble factor, not antagonized by MSH (Fig. 7B), triggering the accelerated degradation of tyrosinase (but not c-met), with similar strength in both B16 cell lines, hence ruling out HGF/SF as a candidate. Also in this case the proteasome pathway was unlikely to be involved, since lactacystin could not prevent the disappearance of tyrosinase (not shown). The labile nature of the diffusible triggering factor is suggested by the fact that its effect on tyrosinase expression is visible in the absence of direct cellular contact, but it is absent in freshly prepared hepatocyte conditioned medium, and even in the soluble fraction of a hepatocyte homogenate (not shown). A weak effect is present, though, in medium conditioned by hepatocytes and melanoma cells in coculture (Fig. 7C), thus suggesting a possible inductive effect by melanoma cells,. The factor appears to be cell type-specific, since it is absent in hepatoma and mesenchymal cells, such as fibroblasts and osteoblasts, independently of their human or murine origin (data not shown). Moreover, liver metastatic colonies produced in vivo by B16 cells are usually pigmented [Rusciano, unpublished observations], hinting again at a labile nature of the factor.

All the known regulators of tyrosinase expression, including lactic acid [Mishima, 1994], operate at the transcriptional level, and therefore our report is the first description of regulatory factors acting posttranscriptionally.

In conclusion, results presented in this paper further our understanding of the regulation of the differentiated phenotype in B16 melanoma cells. Interestingly, we found that HGF/SF and MSH, two factors known to be able to influence the metastatic phenotype of melanoma cells [Rusciano et al., 1998; Bennett et al., 1986], may act antagonistically on the regulation of gene expression. We also describe a marked

downregulatory effect, however limited to tyrosinase expression, by hepatocytes in coculture with B16 cells. This activity, also at the posttranscriptional level, is much stronger than the one exerted by HGF/SF, and seems to be due to some unstable soluble factor(s) produced by hepatocytes.

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