Ectopic expression of *MITF*, a gene for Waardenburg syndrome type 2, converts fibroblasts to cells with melanocyte characteristics

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MITF (*microphthalmia*-associated transcription factor) encodes a transcription factor with a basic-helix-loop-helix-zipper (bHLH-Zip) motif. *MITF* mutations occur in patients with Waardenburg syndrome type 2, a disorder associated with melanocyte abnormalities. Here we show that ectopic expression of *MITF* converts NIH/3T3 fibroblasts into cells with characteristics of melanocytes. *MITF* transfectants formed foci of morphologically altered cells, which resemble those induced by oncogenes, but did not exhibit malignant phenotypes. Instead, they contained dendritic cells that express melanogenic marker proteins such as tyrosinase and tyrosinase-related protein 1. Most cloned cells of *MITF* transfectants exhibited dendritic morphology and expressed melanogenic markers, but such properties were not observed in cells transfected with closely related *TFE3* cDNA. Our findings indicate that *MITF* is critically involved in melanocyte differentiation.

Although some molecules such as *Steel* factor and its receptor are known to be involved in survival and/or proliferation of melanocytes, the molecular mechanisms of melanocyte differentiation were not well understood until the mouse gene for the *microphthalmia* (*mi*) locus, was identified. Dermal, cochlear and ocular melanocytes are known to differentiate from their precursor cells in the neural crest and/or the eye anlage. Hence, disruption of the genes involved in melanocyte differentiation predicts phenotypes related to pigmentary anomaly of skin, ear and eye. Indeed, mutations in several *mi* alleles have been characterized[1,2]: typical phenotypes of mice with homozygous *mi* mutations include white coat colour, small eye and hearing impairment[3,4], each of which has been attributed to melanocyte depletion in the affected organs[4]. Therefore, *mi* is most likely involved in melanocyte differentiation in mice.

As these mouse phenotypic changes are reminiscent of those observed in a subset of individuals with Waardenburg syndrome (WS) type 2 (WS2), mice with mutations at the *mi* locus represent a valuable animal model of this syndrome[5]. WS is a hereditary auditory-pigment syndrome showing clinical and genetic heterogeneity[6]. Persons with WS type 1 (WS1; ref. 7) have dystopia canthorum[6], an outward displacement of the inner canthi of eyes, which is not observed in individulas with WS2. WS is occasionally associated with other abnormalities[8], such as anomalies in the musculoskeletal system of the limb (Klein-WS or WS3; ref. 9) or Hirschsprung disease (Waardenburg-Shah syndrome or WS4; ref. 10). Loss-of-function mutations of a paired box and homeobox gene, *PAX3*, have been found in WS1 and WS3 (refs 11–16). Individuals with WS2 have symptoms which include white patches of the skin, pigmentary disturbances of iris and deafness, but do not show dystopia canthorum. Thus, in contrast with other types of WS, all WS2 symptoms may be explained by melanocyte depletion, the phenotype found in mice with loss-of-function mutations at the *mi* locus.

While mutations of *PAX3* were not found in WS2 (ref. 17), mutations of the *MITF* (*microphthalmia*-associated transcription factor) gene[16,18,19], the human homologue of mouse *mi*[20], are associated with affected members of a subset (WS2A) of WS2 families[16,18,19]. *MITF* and *mi* encode proteins with the basic-helix-loop-helix leucine zipper (bHLH-Zip) motif[1,20]. As mice with mutations at *mi* alleles and individuals with WS2 lack melanocytes in the affected organs[4,21], we speculated that *MITF* could be involved in mediating melanocyte differentiation by functioning as a transcription factor. Here we demonstrate that stable transfection of NIH/3T3 cells with *MITF* induces melanocytic phenotypes.

Morphological changes of *MITF* transfectants

We chose NIH/3T3 cells to examine the function of the *MITF* gene, as these embryonic fibroblasts are known to differentiate into myoblasts or adipocytes in response to ectopic expression of specific lineage determinating transcription factor genes[22–24]. Cells were transfected with an expression vector (pCEV27; ref. 25) containing *MITF* cDNA and, stable transfectants were selected with G-418. These stable transfectants contained refractile cells, often with dendritic processes, which resembled melanocytes (Fig. 1*a*). Although pigmented granules were not visible under a light microscope, expression of tyrosinase, an enzyme involved in melanin biosynthesis

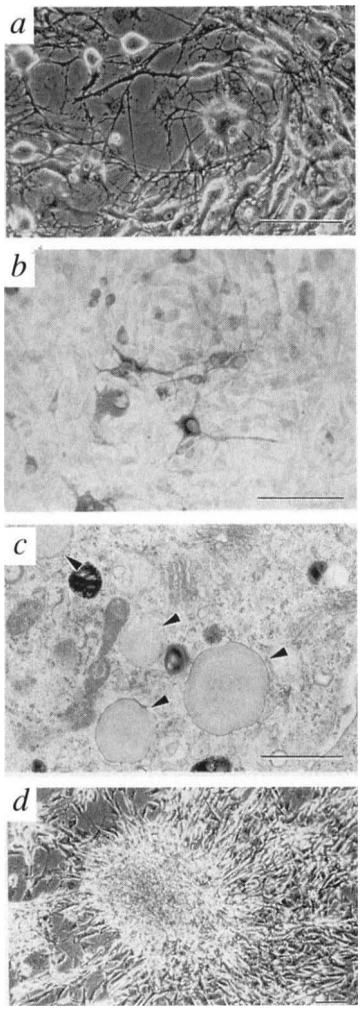

Fig. 1 Cytology of NIH/3T3 cells transfected with an *MITF* expression vector. *a*, A phase contrast micrograph of dendritic cells in *MITF* transfectants. *b*, Immunocytochemical detection of tyrosinase in *MITF* transfectants. Dendritic cells in the transfectants are immunostained. *c*, Electron microscopic view of membrane-bound vesicles (arrowheads) in *MITF* transfectants. No such structure was observed in control vector-alone transfectants. *d*, A focus observed in *MITF* transfectants. Scale bars, 100 μm for *a* and *b*, and 50 nm for *c*.

was observed using immunocytochemistry (Fig. 1*b*). Electron microscopic examination of these cells revealed membrane-bound vesicles with homogeneous matrices (Fig. 1*c*). Such structure is reminiscent of i) immature melanosomes, which are usually observed in albino animals, and ii) the structure observed in Swiss 3T3 cells and L cells transfected with tyrosinase cDNA[26,27]. The high electron density of membrane-bound vesicles in these cells transfected with tyrosinase cDNA is consistent with that of melanin[26,27], while those of NIH/3T3 cells transfected with *MITF* cDNA showed lower electron density. Since NIH/3T3 cells, which are derived from Swiss mice[28], possess the mutated tyrosinase gene[29], the vesicles in *MITF* transfectants are unlikely to contain melanin. Consistent with this notion, biochemical assays[30] did not detect significant activity of melanin synthesis in *MITF* transfectants (not shown) despite immunohistochemical evidence of tyrosinase protein expression (Fig. 1*b*).

Induction of melanogenic marker proteins

As *MITF* transfectants included cells morphologically similar to melanocytes, we examined expression of melanogenic markers known to be involved in melanin biosynthesis[31–33]. Immunocytochemical analyses revealed the expression of tyrosinase (Fig. 1*b*) and TRP1 (not shown) in *MITF* tranfectants, notably in the dendritic cells. Therefore, expression of melanogenic marker proteins was analysed further by western blot analyses of *MITF* transfectants; three marker proteins: tyrosinase, tyrosinase-related protein 1 (TRP1) and TRP2, were expressed (Fig. 2*a*). Unexpectedly, TRP2 expression was also detected in vector transfectants, implicating that it is expressed constitutively in NIH/3T3 cells. Although the expression level of each protein in the transfectants were less than that found in melanocytes or melanoma cells (not shown), these proteins were expressed consistently in independently isolated *MITF* transfectants. The low level of expression of these melanogenic marker proteins may be related to the finding that only a minor fraction (approximately 2%) of *MITF* transfectant cells showed a melanocyte-like appearance. *MITF* transfection in the absence of marker selection led to the appearance of foci of morphologically altered cells which superficially resembled foci induced by oncogenes. However, *MITF* tranfectants did not exhibit the malignant phenotype. They failed to form colonies in soft agar and did not induce tumours in nude mice (not shown). Conversely, the expression of tyrosinase and TRP1 proteins was not induced by transfection of NIH/3T3 cells with several transforming (focus forming) genes, including c-*sis* and *ost*[34] (data not shown), indicating that expression of these melanogenic markers could not be attributed to the focus forming activity of *MITF*.

We found that expression of tyrosinase and TRP1 induced by ectopic expression of *MITF* was influenced by culture conditions. When expression levels of these proteins were compared in low and high (confluent) density cultures, the levels of both marker proteins were higher in cells at saturation density than in growing cells (Fig. 2*b*). In contrast, the level of TRP2 was not influenced by the culture condition. Serum starvation increased expression of tyrosinase (Fig. 2*c*) and TRP1 (not shown) as early as 12 hours after initiation of starvation, and their expression remained elevated by several fold for at least 64 hours.

Detection of melanogenic marker RNAs

We detected expression of tyrosinase mRNAs in *MITF* transfectants by northern blot analysis, although it was lower than that in a mouse melanocyte cell line (melan-*a*) and a mouse melanoma cell line (B16F10) (Fig. 3*a*). Since the RNA expression levels of *TRP1* and *TRP2* were lower than that of tyrosinase in *MITF* transfectants, we utilized reverse transcription (RT)-PCR to detect *TRP1* and *TRP2* mRNA expression. By this method, the expression of *TRP1* was detected in *MITF* transfectants but not in control (vector alone) transfectants (Fig. 3*b*). In contrast, RNA expression of *TRP2* was observed in both *MITF* transfectants and control transfectants (Fig. 3*b*), which agreed with immunoblot and immunocytochemical data (not shown), suggesting the constitutive expression of *TRP2* in NIH/3T3 cells. Expression levels of *TRP1* and *TRP2* mRNAs were lower than that of tyrosinase mRNA, while protein levels of tyrosinase,

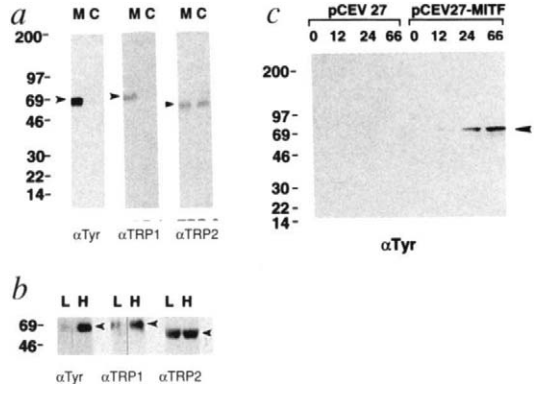

Fig. 2 Immunoblot detection of melanogenic markers. *a*, Immunoblot detection with antisera specific to tyrosinase (αTyr), tyrosinase related protein-1 (αTRP1) or tyrosinase related protein-2 (αTRP2) in lysates of *MITF* transfectants (M) and control vector transfectants (C). Tyrosinase and TRP1 was detected as a single band (arrowheads) only in *MITF* transfectants, while TRP2 was observed in both *MITF* transfectants and vector alone transfectants. *b*, Expression of tyrosinase and TRP1 in *MITF* transfectansts was lower in culture of low density (L) than of high density (H). Sizes of proteins detected in *MITF* transfectants are as follows: tyrosinase ~69 kD; TRP1, ~69 kD; and TRP2, ~67 kD. *c*, Effects of serum starvation on expression of tyrosinase (arrowhead) in *MITF* transfectants (pCEV27-MITF) and vector alone transfectants (pCEV27). Numbers at the top of each lane represent hours after the initiation of serum starvation.

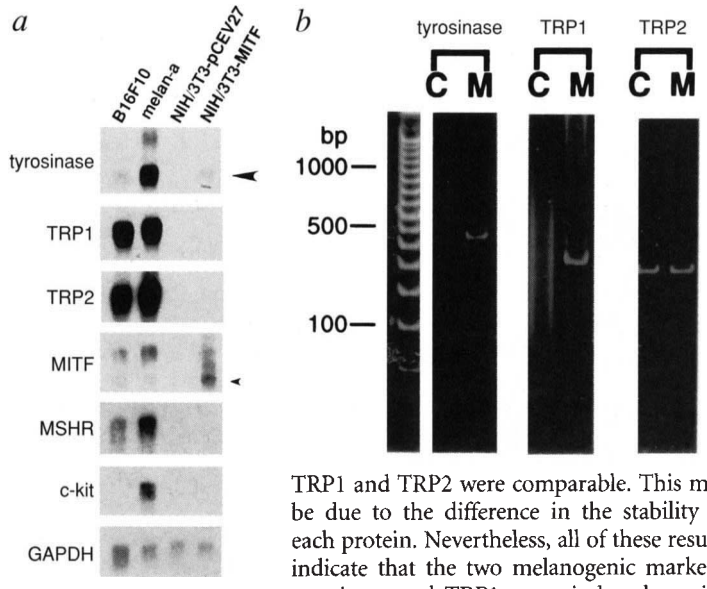

Fig. 3 RNA expression of melanogenic markers as detected by northern blotting (*a*) and RT-PCR (*b*). *a*, The tyrosinase and major *MITF* transcripts in cells transfected with *MITF* are indicated by large and small arrowheads, respectively. The size of transcripts detected in B16F10 and melan-*a* is as follows: tyrosinase, 6.6 and 2.1 kb; TRP1, 2.6 kb; TRP2, 2.4 kb; MITF, 5.5 kb; MSHR (melanocyte stimulating hormone receptor), 4.0 kb; c-kit, 8.2 kb; and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 1.7 kb. Induction of c-kit and MSHR by *MITF* transfection was not detected under these conditions. *b*, RT-PCR. The PCR products for tyrosinase, TRP1 and TRP2 were detected in *MITF* transfectants (M). The product for TRP2 was also detected in control vector transfectants (C).

TRP1 and TRP2 were comparable. This may be due to the difference in the stability of each protein. Nevertheless, all of these results indicate that the two melanogenic markers, tyrosinase and TRP1, were induced specifically by *MITF* expression in NIH/3T3 cells.

Related *TFE3* lacks similar effects

MITF protein is most closely related to three bHLH-Zip proteins; TFEB, TFE3, and TFEC[35–37]. Mi protein has been shown to bind DNA as a homodimer or heterodimer with these proteins[38]. To examine if these MITF-related bHLH-Zip proteins have functions similar to MITF, we transfected NIH/3T3 cells with *TFE3* cDNA. In this series of experiments, we cloned the transfectants by a dilution method[39]. In four out of five clonal *MITF* cell lines, cells were dendritic in shape and resembled cultured mouse melanocytes (Fig. 4*a*, *b*), while the remaining clonal cell line did not show such characteristics. Moreover, cells from these four clonal cell lines were immunostained for tyrosinase and TRP1, as were cultured melanocytes (Fig. 5*a*, *b*, *e*, *f*); the absorption test revealed that the antigen efficiently blocked the immunocytochemical detection (Fig. 5*a*, *e*, inset). In contrast to *MITF* transfectants, all of eleven clonal *TFE3* cell lines failed to show such characteristics (Figs 4, 5), indicating that TFE3 is not capable of inducing melanocyte differentiation by itself.

Discussion

We have shown that transfection of *MITF* cDNA, which encodes a bHLH-Zip protein, can convert NIH/3T3 fibroblasts to cells with a melanocyte-like morphology. These cells also exhibit specific induction of melanogenic markers, tyrosinase and TRP1. As these cells did not show melanin synthesis despite of their melanocytic morphology and expression of melanogenic markers, they are similar to amelanotic melanocytes. Melanin synthesis, however, is not prerequisite for a melanocyte, and amelanotic melanocytes are functional at least in the cochlea[40,41]. Therefore, it appears that *MITF* is critically involved in melanocyte differentiation. In contrast, stable expression of TFE3, a ubiquitous protein structurally related to MITF[35,38], in NIH/3T3 cells did not induce melanocytic phenotypes. Therefore, TFE3 itself is unlikely to be capable of inducing melanocytic differentiation.

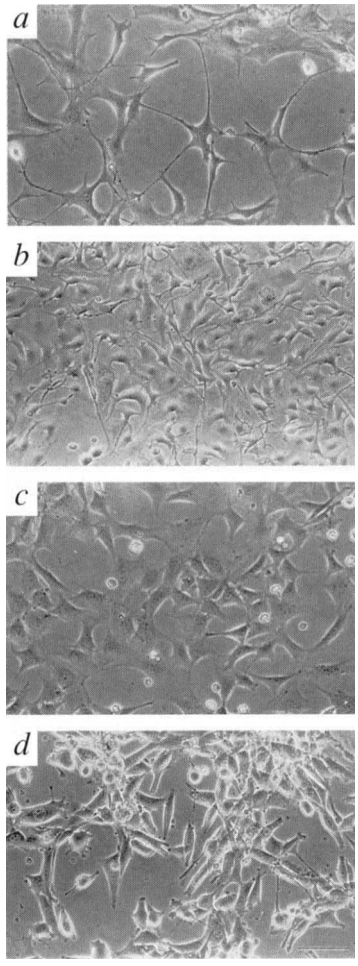

Fig. 4 Morphology of cloned transfectants (*a*, *c*, *d*) and cultured mouse melanocytes (melan-*a*) (*b*). Note the dendritic cell shape of *MITF* transfectants (*a*) similar to that of melan-*a* cells (*b*). *TFE3* transfectants (c) and vector alone transfectants (*d*) did not show such the phenotype. Scale bar, 100 μm.

Although the molecular mechanism for induction of dendritic morphology and melanogenic marker expression by ectopic expression of MITF needs to be clarified, the results of several biochemical studies are consistent with the induction of this phenotype. For example, reporter assays have revealed that transient expression of Mi/MITF *trans*-activates promoters of the tyrosinase and *TRP1* genes in NIH/3T3 cells[19,42–44]. The increased expression of melanogenic marker proteins in *MITF* transfectants was achieved by depriving the transfectants of serum, which contains a number of growth factors, and may have biological implications. Growth and differentiation are usually mutually exclusive, and antagonism between these two states is observed in cultured melanocytes[45] and melanoma cells[46]. Such antagonism is also seen in myogenic differentiation induced by the bHLH proteins of the myogenic gene family[47].

The efficiency of induction of melanocytic differentiation by *MITF* seems to depend on the parental cell line, since we did not detect the expression of melanogenic markers in two other mouse cell lines (10T1/2 and L cell) transfected with *MITF*. At least two interpretations are possible for these observations. Variability of induction has been noted in the case of myogenic differentiation induced by the expression of *myoD*: the induction rate was higher in 10T1/2 cells than in

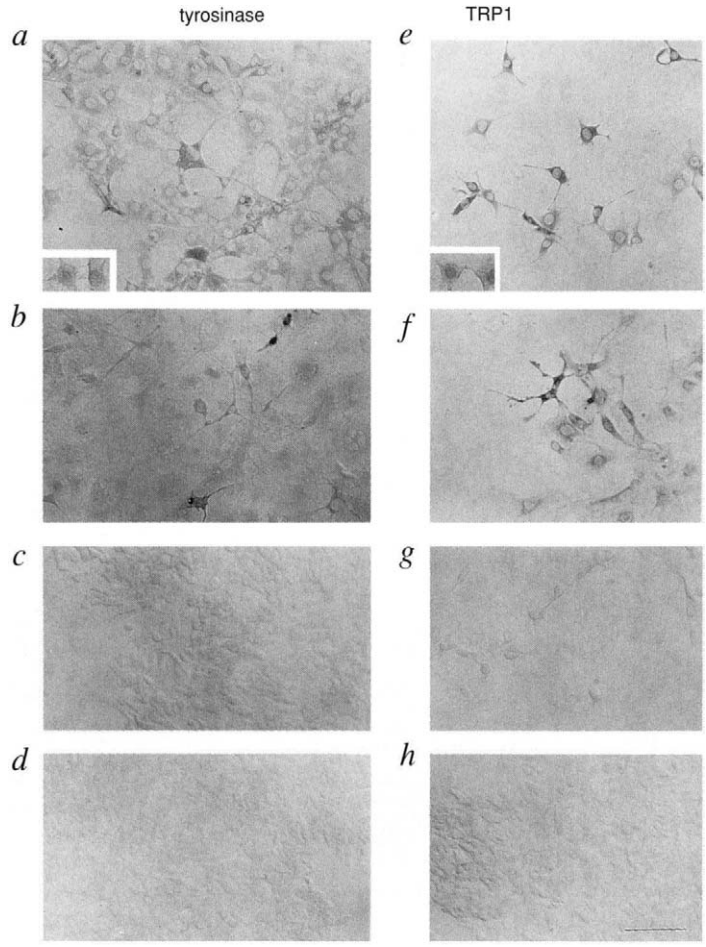

Fig. 5 Immunocytochemical detection of proteins of tyrosinase (*a–d*) and TRP1 (*e–h*) in cloned transfectants and cultured mouse melanocytes (*melan-a*). Immunostaining for both tyrosinase and TRP1 were observed in the cytoplasm of *MITF* clone (*a, e*) as well as in that of melan-*a* cells (*b, f*). Such staining was not observed when antisera, which was absorbed with each antigenic peptide, was used (*a, e*; insets). In contrast to *MITF* transfectants, neither tyrosinase nor TRP1 was expressed in *TFE3* clone (*c, g*) and vector-alone transfectants (*d, h*). Scale bar, 100 μm.

NIH/3T3 and L cells[22]. Interestingly, somatic cell hybrids between 10T1/2 cells and karyotypically normal primary fibroblasts fail to induce myogenic conversion, while transfer of chromosome 11, containing the *MyoD* locus, into 10T1/2 cells results in activation of *MyoD* and myogenic conversion[48]. These studies have suggested that fibroblasts contain an active component capable of inhibiting myogenic differentiation by *MyoD*. Based on these observations, it was recently found that forced expression of a homeobox gene, *MSX1*, in 10T1/2 cells which do not express this gene endogeneously, represses *MyoD* transcription[49]. A similar repression mechanism may exist in the induction process of melanocytic differentiation by *MITF* in some cell lines. Indeed, *trans*-activation of the fibroblast tyrosinase gene has been shown to depend on the absence of a putative 'extinguisher gene'[48–50], which presumably is responsible for the extinction of pigmentation in somatic cell hybrids between melanoma cells and non-pigment cells such as sublines of L cells, NCTC 2555 cells, and hepatoma cells[50–53]. Success or failure of induction of tyrosinase by *MITF* in several cell lines in this study may depend on the expression level of the extinguisher gene or similar repression genes. Alternatively, melanocytic differentiation may require a factor which promotes TRP2 expression or the constitutive expression of TRP2 itself. The finding that a subset of WS2 families do not map to the *MITF* gene[54] may suggest the involvement of other genes in melanocyte differentiation.

Transcription factors with bHLH motifs are associated with cell fate determination in vertebrates[22,23,55,56] and invertebrates[57–59]. Some proteins with bHLH-Zip motif are likely to have similar functions. For example, ADD1 is associated with adipocyte determination and differentiation[24]. Our data show a link between expression of MITF and melanocyte differentiation. We have previously shown that the mutated *MITF* gene found in affected members of two WS2 families cannot *trans*-activate the tyrosinase promoter[19]. These data are consistent with the concept that loss-of-function mutations in the *MITF* gene causes the failure of such differentiation, leading to the WS2 phenotype.

Methods

Transfections. NIH/3T3 cells were transfected with varying amounts (0.01 to 1.0 μg) of the eukaryotic expression vector pCEV27 (ref. 25) or pCEV27 containing, either human *MITF* cDNA (melanocyte-specific isoform[1]) cloned from a melanocyte cDNA library[20], *ost* cDNA[34], c-*sis* cDNA or *TFE3* cDNA[35] (a gift of T. Kadesh) as described[60]. Stable transfectants were selected for G418-resistance. For the cloning of cell lines, standard dilution method[39] was used.

Detection of melanogenic marker proteins. For immunocytochemical analyses, cells were fixed with 4% paraformaldehyde, permeabilized by 0.1% Triton X-100 in PBS containing 10% normal goat serum, incubated with diluted antisera (1: 1,000), immunostained using Vectastain ABC Elite kit and visualized by diaminobenzidine reaction. For immunoblot detection, lysates (15 or 40 μg of protein) of NIH/3T3 transfectants were analysed on 8–16% gradient polyacrylamide gel. Following electrophoresis, gels were electroblotted to nitrocellulose membranes. The blots were probed with antisera[31–33] to tyrosinase, TRP1 or TRP2 (gifts of V.J. Hearing), and the bands were visualized by chemiluminescence. These antisera detect each antigen specifically[31–33]. For the absorption test, antisera incubated at 4 °C overnight with each antigen (1 mg/100 μl), a peptide derived from carboxy termini of mouse tyrosinase, TRP1, or TRP2, was used. For the serum starvation studies, cells were seeded at a density of 1×10^3 cells per well in duplicate 60 mm culture dishes and cultured in DMEM with 10% calf serum. After 12 h the medium was switched to the serum starvation medium, a 1:1 mixture of DMEM and NF40 containing 5 μg/ml of transferrin and 10 mM sodium selenium. At the indicated times (in h), samples were obtained and cell lysates (30 μg protein/lane) were analysed for tyrosinase expression using western blot technique.

Detection of melanogenic marker mRNAs. For northern blot analyses, 5 μg of total RNA extracted from the transfectants were electrophoresed through a formaldehyde agarose gel, transferred to nylon membranes, and hybridized with ^{32}P-labelled probes[61].

For RT-PCR amplification, total RNA was extracted from *MITF*-transfectants, control vector transfectants and melan-*a* cells using TriReagent (MRC) according to the manufacturers' instructions. Eight μg of total RNA was used for each amplification. RNA was reverse-transcribed using oligo dT_{12-18} as a primer and RNase H$^-$ reverse transcriptase (Gibco BRL). Amplification was carried out using 10 pmol of each primer in a reaction volume of 50 μl. The sets of upper and lower primers used to detect the expression of tyrosinase, TRP1 and TRP2 were 5'–GGGCCCAAATTGTACAGAGAAGC–3'/5'–CTGCCAGGAGAAGAAGGATG–3', 5'–GCCCCAACTCTGTCTTTTCTCAAT–3'/5'–GATCGGCGTTATACCTCCTTAGC–3' and 3'–GG-

ACCGGCCCCGACTGTAATC–3'/5'–GTAGGGCAACG-CAAAGGACTCAT–3', respectively. Samples were heated 95 °C for 3 min, and amplified by 40 cycles of 94 °C for 1 min, 51 °C for 1 min and 72 °C for 1 min, with a final extension step of 1 min at 72 °C. PCR products in 10 µl of the reaction mixture were run on 4–20% gradient polyacrylamide gels and stained with ethidium bromide. The molecular sizes of PCR products obtained from transfectants were the same as those obtained from melan-*a* cells.

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