Pax3 Down-regulation and Shut-off of Melanogenesis in Melanoma B16/F10.9 by Interleukin-6 Receptor Signaling*

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The microphthalmia-associated transcription factor (Mitf) is essential for melanocytic lineage development and for expression of melanogenic enzymes, such as tyrosinase. Interleukin-6 receptor/interleukin-6 chimera (IL6RIL6) induces in B16/F10.9 melanoma cells a loss of melanogenesis preceded by a sharp decrease in Mitf mRNA and gene promoter activity. In the Mitf promoter, the main cis-acting element mediating the IL6RIL6 effect is shown to be the binding site of Pax3, a paired homeodomain factor regulating among other things the development of melanocytes. Pax3 protein and mRNA levels decline steadily after IL6RIL6 treatment, and overexpression of an ectopic Pax3 cDNA suppresses the Mitf promoter inhibition. Loss of the synergism between Pax3 and Sox10, a high mobility group domain costimulatory factor, seems to be critical in the rapid decrease in Mitf gene expression. The Pax3 down-regulation in IL6RIL6-induced F10.9 cells is linked to growth arrest and transdifferentiation to a glial cell phenotype. IL6RIL6 stimulates the interleukin-6 family cytokine receptor gp130, leading to the rapid phosphorylation of Stat3 on tyrosine 705. This phosphorylation is required for Pax3 down-regulation and Mitf promoter silencing since these are inhibited in F10.9 cells overexpressing the Stat3 DN-mutant Y705F.

Vertebrate melanocytes derive from multipotent neural crest cells, which are also the source of peripheral neurons, Schwann cells, and other glial cells (for review, see Ref. 1). In the skin, melanoblasts reach the epidermis, where they differentiate and synthesize melanin in melanosomes that are transferred through dendritic cell processes to surrounding keratinocytes. The melanocytic program of gene expression is regulated in large part by the bHLH-Lz microphthalmia-associated transcription factor (Mitf)1 (2, 3). Mitf is essential for melanoblast survival (4) and for expression of melanocytic characteristic (5). Mitf target genes include enzymes which regulate the synthesis of melanin, mainly tyrosinase (6) and tyrosinase-related protein-1 (7). The transcription factors Pax3, Sox10, and LEF-1 transactivate the Mitf gene promoter and play important roles in melanocytic lineage development (8–10). A CAMP response element (CRE) in the Mitf promoter mediates a transient induction of Mitf transcription in response to the α-melanocyte-stimulating hormone, α-MSH (11, 12). In the differentiated melanocytes, the synthesis of melanin and growth of short dendritic arborizations releasing melanosomes is increased by UV light (13). The effect of UV light is mediated by up-regulation of α-MSH, activation of the G protein-linked MC1 receptor, and stimulation of cAMP formation (for review, see Ref. 14).

Malignant melanoma cells commonly retain differentiated characteristics of the melanocytic lineage (15), including the regulation of melanogenesis and dendritic differentiation by UV light (16) as well as by α-MSH (17). Another type of differentiation is seen in senescent melanocytic nevus cells in the dermis, where melanogenesis is lost, and the cells adopt the spindloid morphology and gene markers of early Schwann cells (18). How the transdifferentiation process operates has not been clarified. This transdifferentiation is not seen in invasive melanoma, although cases of melanomas displaying Schwann cell-like features have been reported (19). Loss of melanogenesis and of tyrosinase can, however, be induced in melanocytes and in melanoma cells by cytokines such as interleukin-6 (IL6) and tumor necrosis factor-α (20, 21).

IL6-family cytokines use gp130 as a common signaling molecule, and the transcription factor Stat3 plays a major role in growth regulation, survival, and differentiation effects of these cytokines (for review, see Refs. 22–24). IL6 in complex with its receptor (IL6R), either membrane-anchored or soluble, or the chimeric molecule IL6RIL6 (25, 26) associate with the transmembrane receptor gp130, triggering gp130 dimerization. This leads to activation of intracellular signaling cascades, including the Janus kinase/STAT pathway, extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK1/2), and phosphatidylinositol 3-kinase (22). Tyrosine phosphorylation of gp130 by Janus kinases stimulates recruitment and phosphorylation of Stat3 at tyrosine 705 (27) and transfer of activated Stat3 to the nucleus. Stat3-transactivating and -signaling functions are regulated by homo- and heterodimerization with STAT family or other factors (28). Activated Stat3 binds to palindromic Stat response element (STAT-RE) on target genes. Optimal activation of transcription by Stat3 requires phosphorylation of Ser-727 in the Stat3a C-terminal activation domain (29).

We previously described a model of metastatic murine melanoma cells, B16/F10.9, which respond to treatment with IL6 and soluble IL6Ra, or with the chimeric molecule IL6RIL6, by arrest of proliferation and transition to an elongated spindloid
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morbidity (26, 30). In the present study, we show that tyrosinase activity and melanogenesis are strongly inhibited in F10.9 melanoma cells treated with IL6RIL6 and describe the molecular mechanisms mediating the silencing of the tyrosinase gene. The major mechanism is a loss of Mitf expression and MITF promoter activity in these cells, itself caused by down-regulation of Pax3. The phosphorylation of Stat3 on Tyr-705 appears essential for the down-regulation of Pax3 and Mitf.

EXPERIMENTAL PROCEDURES

Cell Culture, Chemicals, and Cytokines—Murine B16 melanoma cells, clone F10.9 (31), were obtained from Dr. L. Eisenbach (Weizmann Institute, Israel). Cells were cultured as a monolayer at 37 °C, 5% CO2, in Dulbecco’s modified Eagle’s medium with 8% fetal calf serum (Biobals, Bet Ha Emek, Israel) supplemented with glutamine, penicillin, and streptomycin and subcultured every 3 days at 10–30% confluency. The chimeric molecule interleukin-6 receptor/interleukin-6 (IL6RIL6 (26)) was produced by recombinant Chinese hamster ovary cells (CHO clone L12). The cells were grown on microcarrier beads, and the soluble IL6RIL6 protein was purified as a single 85-kDa species by immunofinity on an Affi-Gel-10 (Bio-Rad) column coupled with anti-IL6R (Mc Ab 34.14) (Interpharm, Israel). To follow up the effect of IL6RIL6 at different times, cells were plated at less than 50% confluency, equal numbers of cells were plated in non-treated and treated wells, and treatment was applied about 2 h after seeding (in general 150 ng/ml IL6RIL6). Stock solutions of 20 μM forskolin (Sigma) were prepared in Me2SO, 1,3,4-Dihydroxyphenylalanine (l-Dopa) (Sigma) stock solution was dissolved in water (80 mg/ml).

Tyrosinase Activity—For tyrosinase activity, measured as the rate of l-Dopa oxidation (32, 33), F10.9 cells were seeded in 6-well Costar plates (5 × 105 cells/well) and cultured for the indicated times. Cells were lysed with 0.1 M phosphate buffer, pH 6.8, containing 1% Triton X-100. Duplicate 40-μl aliquots of 1300 × g supernatants (or lysis buffer only) were distributed on microtiter plates and supplemented with 100 μl of L-Dopa solution (2 mg/ml in phosphate buffer). The A at 570 nm was measured in triplicate wells with automatic blank subtraction. Absorbance values were corrected for protein concentration.

Northern Blots and RT-PCR—F10.9 cells were seeded at less than 50% confluency and either treated with 150 ng/ml IL6RIL6 or left untreated. Total RNA was extracted with Tri-Reagent (Molecular Research Center, Cambridge) as recommended by the manufacturer.

For Northern blots, RNA samples (15 μg/lane) denatured in 50% formamide and 6% formaldehyde were resolved by electrophoresis in 1% agarose gels in 0.5x MOPS/acetate buffer with 6% formaldehyde and transferred to nylon Hybond N (Amersham Sciences). DNA probes labeled with [32P]dCTP by random priming with the Rediprime II kit (Amersham Biosciences) were hybridized to RNA blots in standard solutions (34) containing 50% formaldehyde and 10% formamide.

For RT-PCR, RNA samples (about 2 μg/assay) were reverse-transcribed with SuperscriptII (Invitrogen) in the presence of oligo-dT in 20 μl, and 2 μl of the RT reaction was used for amplification with Taq polymerase. The primers used to amplify specific mouse cDNAs are listed below. Mitf (GenBank™ accession number NM_008601): forward (F) nucleotides 82–101, reverse (R) 1382–1401. Pax3 (NM_008781): F 414–438; R 857–861. For quantification of Pax3 isoforms, mRNAs (35): F 850–880; R (Pax3c) 1735–1759; R (Pax3c, Pax3d, and shorter isoforms) 2224–2253. Tyrosinase (D00131): F 430–454; R (Pax3 mut1) 1382–1401. The original PCR fragment was obtained by exonuclease III digestion on the 5′ end and the plasmid sequence. Glyceraldehyde-3-phosphate dehydrogenase (G3 PDH) primers (CLONTECH) were used to verify RNA loading. Amplification conditions were 94 °C (1 min), 52–58 °C (30 s), 72 °C (2 min) for 25 cycles or, in specific cases, 20 cycles (G3 PDH) or 24 cycles (Mitf, tyrosinase). Sequencing of PCR fragments was done on DNA analyzer 3700 (PE Applied Biosystems, Hitachi).

Western Blots—F10.9 cells or transformed clones seeded at less than 50% confluency were either treated with IL6RIL6 or left untreated. At the end of the incubation period, cells washed with phosphate-buffered saline were lysed with radioimmune precipitation buffer (34) containing protease inhibitors (Calbiochem), or nuclear proteins were extracted (30). Cell extracts were analyzed by SDS-PAGE, and proteins were transferred to nitrocellulose membranes Ptoiran Ba55 (Schleicher and Schuell). For immunodetection, the blots were first blocked with a solution of 5% (w/v) low-fat milk in phosphate-buffered saline, 0.5% Tween 20 (1 h, room temperature), then incubated with specific rabbit antibody diluted in phosphate-buffered saline/triton (i.e., 2% milk, 2 h, room temperature), washed with goat antibody conjugated with horseradish peroxidase-conjugated immunopurified IgGs (Jackson Immunoresearch). Antibody binding was revealed with Pierce ECL reagents. Rabbit anti-sox10 antibody (CellMines) and anti-Pax3 antibody (Geneika Biotechnology Inc.) were used at 1/2000 dilution, and rabbit IgG against CREB/ATF1 (Biobals) was used at 1/1000 dilution.

With transfection vectors—RT-PCR with F10.9 total RNA was used to amplify coding sequences corresponding to Pax3c cDNA (35/36, 35871: nucleotides 255–1759) and Sox10 cDNA (AF04743: nucleotides 39–1339). PCR products were cloned in the PGEM EMBL vector (Promega) and sequenced. In the Pax3 Δ-Cterm construct, the Pax3 open reading frame ends at codon 277, after the DNA binding site. For expression the cDNAs were cloned in the pcdNA3 vector (Invitrogen). pcdNA3-MITF contains the human MITF coding sequence (a gift from Dr. E. Rumin, Hadassah Medical School). The CREB/ATF1-binding protein in pcPE4 expression vector was a gift from Dr. Roni Segel (Weizmann Institute, Israel). The mouse Stat3a/krf cDNA cloned in the EcoRI site of pc-EGFP. p-cEGFP was prepared by replacing the NeoR sequence (between Smal-Scal sites) of pcdNA3 (Invitrogen) by the coding sequence of the green fluorescent protein (GFP) (fragment Smal-AfIII) from pEFGP-N1 (CLONTECH).

Genomic DNA Reporter Gene Constructs—PCR amplification was used to isolate a fragment of the mouse tyrosinase gene upstream sequence, including TATA box and promoter region (D04398: F 2019–2031, R 2052–2070). The fragment was cloned into the XhoI site of the pG3-bis vector (Promega) to produce the plasmid tyrosinase-Luc. Three repeats of the sequence 5′-gactaAGTTTACTATGCTCTT corresponding to MITF-responsive sequence (M-box) in the tyrosinase gene were cloned in the BgII site of the pG3 promoter plasmid (Promega). A plasmid containing the human MITF promoter region (38) and mutated sequences 2377–3180 of pGL2-basic vector (Promega) was a gift from Dr. Ballotti (Nice, France) (11). MITF sequences 1161–2356 (or 1097–99, relative to the start site) were amplified by PCR and cloned at the BgII site in pG3-bis. From this construct, deletions between XhoI (in polylinker) and either PstI or NsiI sites (in MITF) created the −381 and −46 constructs. Other deletions were obtained by exonuclease III digestion on the −381/−99 construct cut with SacI or NdeI, followed by mutating the remaining delete sites (NdeI, SacI, and NdeI deletion deletion, Stratagene). The fragments resulting from HindIII digestion were recombined in pG3-bis with XhoI (blunt end) and HindIII to create constructs −318, −277, −246, and −192. Three contiguous internal fragments between AatII and EcoRV, EcoRV and NsiI, or NsiI and DdeI were excised from the sequence −381/−99, respectively, in constructs −146/−122 (CREI-del), −119/−46, or −46/−51/Pax3del2. In the LEF-1del construct, the fragment −234 to −192 was replaced by 40 base pairs of linker sequence. To produce the −381/−46 thymidine kinase construct, the fragment −381/−46 was amplified by PCR and cloned in front of minimal herpesvirus thymidine kinase promoter sequence (AF057310, nucleotides −148/−1) cloned in the BgII site of pG3-bis. A plasmid containing eight repeats of the Pax3 site in front of the mitf promoter (−31/−99) was prepared as described (8).

Mutations were performed by two-step PCR to change the sequence ATTAATACGTAGG (−260/−244), containing Pax3 paired and homedomain binding sites, into AggATACATTCGAGC (Pax3 mut1). The sequence TACGTCCT (−147/−141), containing the CRE site, was mutated to TGAgTCA in CRE mut. The sequence TTCGCTAAAG (−266/−277), containing C/EBP/gp130-IL6 binding site, was mutated to TTCGTCgAG in the C/EBP mutant construct. Mutations were all in the context of the −381/−99 sequence.

Cell Transfections—For transfections, F10.9 cells in the log phase of growth were seeded in 6-well Costar plates (2 × 105 cells/well). After 24 h, each well received 1.2 ml of mixture containing 2 μg of plasmid DNA, including 0.5 μg of plasmid pSV2-galactosidase (Promega) or pCMV-galactosidase and 10–12 μl of LipofectAMINE (Invitrogen) in
RESULTS

Effects of IL6RIL6 on the Melanocytic Phenotype of B16/F10.9 Melanoma Cells—Tyrosinase is a rate-limiting enzyme for the synthesis of melanin pigments in melanocytes and melanoma cells (39). Whereas in untreated cultures of B16/F10.9 cells the tyrosinase specific activity increased with time, treatment of the cells by IL6RIL6 caused a gradual loss in the melanogenic enzyme (Fig. 1A). Melanocytic differentiation can be enhanced in melanomas by agents that increase intracellular cAMP, such as forskolin (17, 40). The F10.9 cells responded to forskolin by a marked increase in tyrosinase, but this increase was also completely prevented by IL6RIL6 (Fig. 1B). Spectrometric measures of melanin released by the cells showed changes similar to tyrosinase, the black coloration of the medium being increased by forskolin and inhibited over 95% by IL6RIL6 addition (not shown). Forskolin produced the typical melanocytic differentiation characterized by formation of a dense crown of short dendritic expansions (Fig. 1C), which are sites of melanosome release. In contrast, the cells treated by IL6RIL6 became elongated and developed long processes often bipolar and branched with thinner ramifications (Fig. 1C), suggesting that the loss of melanogenesis is part of a differentiation toward another phenotype.

Inhibition of Tyrosinase and MITF Genes Transcriptional Activity by IL6RIL6—Analysis of tyrosinase mRNA in Northern blots revealed that the mRNA for MITF, a factor of critical importance for tyrosinase gene transcription (6) and a marker of the melanocytic lineage (for review, see Ref. 3), was suppressed by IL6RIL6 already 6 h after treatment (Fig. 2). To examine the transcriptional activity of the tyrosinase gene, a 550-base pair promoter segment linked to a luciferase reporter gene was transfected into the F10.9 cells (Table I). IL6RIL6 produced an 80% decrease in the activity of the tyrosinase promoter measured 72 h after treatment. Forskolin increased the tyrosinase promoter activity, but IL6RIL6 inhibited it even in the presence of forskolin (Table I). Because the target of MITF in the tyrosinase promoter is an M-box element (6, 11), we tested a luciferase gene driven by repeats of the M-box and found that its expression is reduced in IL6RIL6-treated cells (Table I). Evidence that these effects may be attributed to a decrease of MITF was obtained by co-transfection with a plasmid expressing the human MITF cDNA; under these conditions, no inhibition by IL6RIL6 was observed with either the M-box or the tyrosinase promoter either with or without forskolin (Table I). In contrast, transfection with the coactivator CREBP-binding protein/p300 did not suppress the inhibitory effect of IL6RIL6 (Table I), although CREBP-binding protein binds MITF and modulates its transcriptional activity (42). However, CREBP-binding protein/p300 also enhances the activity of factors binding to cAMP response elements (for review, see Ref. 43) and enhanced the tyrosinase promoter activity in the presence of forskolin (Table I).

We next examined whether IL6RIL6 affects the transcriptional activity of the MITF gene. Reporter genes containing the 5’-flanking promoter sequences (38) demonstrated about 80% inhibition in the IL6RIL6-treated F10.9 melanoma cells (Table II). Similar levels of inhibition were observed with 5’-deleted MITF promoter fragments ranging from −1907/+99 down to −277/+99. An MITF promoter segment in which the TATAA box and further 3’ sequences were deleted and replaced by the minimal hamster sarcoma virus thymidine kinase promoter also showed significant inhibition by IL6RIL6 (construct −381/−46 in Table II).

MITF Promoter Elements Involved in the Repression by IL6RIL6—The MITF promoter between positions −268 and −141 is the target of multiple regulatory elements (see Fig. 3). A site positively regulated by the paired homeodomain factor Pax3 is located between −260 and −244 (8). Immediately upstream is a sequence (−268/−262) regulated by Sox10, a high mobility group-box protein that cooperates with Pax3 (9, 44). At position −199/−193, there is a functional binding site for β-catenin-regulated LEF-1 (10). At −147/−141 is the CRE mediating the induction of MITF by forskolin (11). A second Pax3 binding site (−40/−20) was proven important for synergism with Sox10 (9).
When we deleted the sequences between −277 and −246 in the MITF promoter, there was a marked decrease in the inhibitory effect of IL6RIL6 (Table II). This deletion had lowered basal activity but was still active in comparison to shorter segments. Mutating the first Pax3 binding site in the context of the −381/+99 segment had the same effect as the −246 deletion and virtually eliminated the influence of IL6RIL6 (Pax3 mul1 in Table II). Internal deletion −46/−31 removing only the second Pax3 site (Pax3del2) reduced basal activity, but the effect of IL6RIL6 was still clear. However, when both Pax3 sites were altered (Pax3 mut1,2), the activity dropped to one of empty vector (Table II). In contrast, a mutation of the C/EBP-Î²NF-IL6 binding site did not reduce all the repression by IL6RIL6, whereas deletion of the LEF-1 site had only partial effects (Table II). Other deletions such as −119/−46, which removes the Oncocut-2 site (45), reduced basal promoter activity, but the inhibition by IL6RIL6 was still of 50–60% (data not shown). Internal deletion −146/−122 (CREdel) or mutating the CRE site (CRE mut) strongly reduced the basal activity of the MITF promoter and decreased the inhibition by IL6RIL6 (Table II). However, further experiments performed in conditions stimulating the CREmut promoter basal activity indicated that the CRE mutation does not prevent inhibition by IL6RIL6 (see Table IV and “Discussion”).

In cells treated with forskolin (Table III), the wild type MITF promoter was more active and was inhibited 5.6-fold by IL6RIL6. The activity of the CREmut was not increased by forskolin and behaved as the control vector. The Pax3 mut1 promoter was stimulated by forskolin and less inhibited by IL6RIL6 (2.7-fold inhibition) than the wild type promoter, indicating again the importance of the Pax3 site.

**Down-regulation of Pax3 mRNA and Protein in IL6RIL6-treated Cells—Immunoblots of F10.9 whole cell extracts revealed a differential decrease of the 54-kDa Pax3 protein starting from 12 h after the addition of IL6RIL6 (Fig. 4A). We did not detect comparable variations in CRE-binding proteins (CREBP or ATF1) in these extracts (not shown). In view of the cooperative effect of Sox10 and Pax3 on the MITF promoter (9), we attempted to determine if Sox10 is decreased together with Pax3. Whereas at 6–12 h a moderate decrease in the 56-kDa Sox10 protein was observed, there was instead an increase relative to non-treated cells at 48–96 h (Fig. 4A). Similar results were obtained when nuclear proteins were analyzed in a blot that was first reacted with anti-Pax3 antibodies and, after stripping, was reacted with anti-Sox10 antibodies (Fig. 4B). Scanning of this blot (Fig. 4C) showed again that Pax3 is gradually decreased, whereas Sox10 decreases only at early times and is increased at late times. This pattern was verified in three experiments with different F10.9 extracts.

A decrease in the mRNA level of Pax3 after IL6RIL6 treatment was similarly seen in RT-PCR using primers common to most Pax3 isoforms (35). Fig. 4D shows that the decrease in Pax3 mRNA correlated well to the decrease in MITF mRNA. Conversely, cells exhibit a concomitant increase in the mRNA for the glial fibrillary acidic protein, one of the glial cell-specific genes induced in F10.9 cells in response to IL6RIL6 (46).2

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**Table I**

| Lucifierase constructs | Expression vector | Non-treated | IL6RIL6 | IL6RIL6 effect | Forskolin | Forskolin + IL6RIL6 | IL6RIL6 effect |
|-----------------------|------------------|-------------|---------|--------------|-----------|-------------------|--------------|
| Tyrosinase-Luc.       | pcDNA3           | 57          | 11      | −80          | 130       | 37                | −71          |
|                       | MITF             | 190         | 203     | +13          | 288       | 346               | +20          |
|                       | CBP              | 53          | 12      | −78          | 241       | 41                | −83          |
| M-box-Luc.            | pcDNA3           | 740         | 446     | −40          |           |                   |              |
|                       | MITF             | 9820        | 12730   | +29          |           |                   |              |

**Table II**

| MITF-Luc constructs | Non-treated | S.D. | IL6RIL6 | S.D. | IL6RIL6 effect |
|---------------------|-------------|------|---------|------|----------------|
| −1097/+99           | 183         | 30   | 21      | 8    | −88            |
| −381                | 100         |      | 20      | 7    | −80            |
| −318                | 120         | 8    | 17      | 5    | −86            |
| −277                | 60          | 12   | 24      | 7    | −60            |
| −246                | 25          | 12   | 22      | 10   | −12            |
| −192                | 15          | 3    | 15      | 6    | NC             |
| −46                 | 2           | 1    | 2       | 2    | NC             |
| C/EBP mut.          | 150         | 30   | 30      | 5    | −80            |
| Pax3 mut1           | 23          | 7    | 20      | 3    | −13            |
| LEF-1del.           | 61          | 19   | 31      | 12   | −48            |
| CRE mut.            | 22          | 10   | 14      | 6    | −36            |
| Pax3 del2           | 30          | 10   | 8       | 4    | −73            |
| Pax3 mut1,2         | 7           | 4    | 8       | 3    | NC             |
| pG13b               | 3           | 1    | 4       | 1    | NC             |
| −381/−46            | 100         |      | 33      | 3    | −67            |
| pG13b-TK            | 25          | 3    | 21      | 3    | −16            |

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Pax3 and Mitf Shut-off by gp130 Signaling

Ectopic expression of MITF reverses inhibition by IL6RIL6 of tyrosinase gene promoter activity or M-box reporter activity

Tyr-Luc contains the mouse tyrosinase gene 5’ sequences (−446/+107) in pG13 basic, and M-box-Luc contains three repeats of the tyrosinase sequences −113/−97 in front of the minimal SV40 promoter. Luciferase and pSV2-galactosidase reporter plasmids (respectively, 1 µg and 0.25 µg/well) were co-transfected in F10.9 cells with empty expression vector pcDNA3 or with expression vectors where MITF or co-activator CBP coding sequences are controlled by a cytomelalgivirus promoter (1 µg/well). Average luciferase activities (normalized on β-galactosidase) of triplicate assays are reported. S.D. vary from 2 to 13% of average. IL6RIL6 effect was calculated as the % of values with IL6RIL6 over values without transfections in duplicate are shown. NC, not changed.

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**Table II**

IL6RIL6 effect on luciferase activity regulated by MITF genomic sequences

MITF-luciferase constructs (schematized in Fig. 3) were transfected into F10.9 cells in several experiments with different plasmid preparations, always including the native construct −381/+99 as a reference. The luciferase activity value (normalized on β-galactosidase) obtained for construct −381/+99 in non-treated cells was taken as 100 and used to normalize other values. For the thymidine kinase (TK)-promoter constructs (last two rows), the luciferase activity of the construct −381/−46 in non-treated cells was taken as 100. The average value and S.D. from four distinct transfections in duplicate are shown. NC, not changed.
Pax3 and Mitf Shut-off by gp130 Signaling

Overexpression of Pax3 Restores MITF Gene Activity—If the impairment of the MITF gene promoter activity were related to the decrease in Pax3 after IL6RIL6 treatment, ectopic expression of Pax3 in these cells would be expected to restore the promoter activity. For these experiments we used one of the major murine isoform, Pax3c, and the expression vector pcPax3c (35) was co-transfected together with the −381/+99 MITF-luc reporter into F10.9 cells. In non-treated cells, increasing amounts of pcPax3c stimulated the MITF promoter activity (Fig. 5A) up to a certain level and then decreased it, typical of Pax3 action (48). Strikingly, in IL6RIL6-treated cells, overexpression of Pax3c at high plasmid doses overrode the inhibitory effect of IL6RIL6 (Fig. 5, A and C). A mutant Pax3Δ-

Cterm failed to overcome the repression by IL6RIL6, indicating the need for Pax3-transactivating domain (Fig. 5C).

Activity of the MITF promoter was also increased by co-transfection with increasing doses of the Sox10 expression vector pcSox10, as expected (9, 49, 50). However, the inhibition by IL6RIL6 remained the same irrespective of the addition of Sox10 (Fig. 5, B and D). We verified that the IL6RIL6-treated cells transfected with the high dose of Sox10 or of Pax3 expression vectors contained the expected increased amount of the respective proteins (Fig. 5E). In particular, the difference in Pax3 levels between IL6RIL6-treated and non-treated cells was abolished by the ectopic Pax3c expression. These results indicate that Pax3, but not Sox10, overcomes the effect of IL6RIL6 on the MITF gene.

Cooperation of Sox10 and Pax3 Is Deregulated in IL6RIL6-treated Cells—When combined at low plasmid doses, the expression vectors of Pax3 and Sox10 had a synergistic effect on the MITF promoter activity in non-treated F10.9 cells (Table IV). In line with previous reports (9), this synergism was much reduced when the −260/−244 Pax3 site was mutated and abolished when the second putative Pax3 site was also removed by a −46/−31 deletion (respectively Pax3 mut1 and Pax3 mut1,2 in Table IV). In contrast, a MITF promoter with a mutation in the CRE site can still be activated by the synergistic combination of Sox10 and Pax3, although by itself this promoter has very low activity (Table IV). In all cases, including the CRE mutant, treatment of the cells by IL6RIL6 strongly reduced the activity of the MITF promoter (Table IV). This shows that the CRE mutant is still sensitive to the inhibitory effect of IL6RIL6 and that the main effect of IL6RIL6 is on Pax3 and its synergistic action with Sox-10. It should be noted that to observe the synergistic effect, relatively low doses of the Pax3 and Sox10 plasmids have to be used so that ectopic Pax3 alone does not override the effect of IL6RIL6. In fact, immunoblots showed that under these conditions, Pax3 was still reduced in the IL6RIL6-treated cells. Nevertheless, the larger inhibition by IL6RIL6 seen when Sox10 is co-transfected together with Pax3 as compared with pcDNA3 control cells (Table IV) attests for some deregulation of the Sox10-Pax3 cooperative effect. Hence, when Sox10 is increased, the repression by IL6RIL6 is emphasized.

The inhibition due to IL6RIL6 was seen also with a reporter gene driven only by eight repeats of the Pax3 binding sequence at −260/−244, placed upstream of a minimal MITF promoter (−31/+99). This reporter had a low basal activity and was stimulated by Pax3 (Fig. 6). Although no consensus Sox10 binding site is present, the reporter was stimulated by Sox10 and showed synergistic activation by Pax3 and Sox10 (Fig. 6). The Sox10-dependent activity of this PaxM8 chimeric promoter was strongly inhibited by IL6RIL6, whereas with Pax3 alone, the inhibition by IL6RIL6 was much lower. Again the synergistic action of Pax3 and Sox10 was very sensitive to inhibition by IL6RIL6 (Fig. 6).
Involvement of Stat3-dependent Signaling in the Down-regulation of MITF and Pax3

Intracellular signaling events mediated by the IL-6-family receptor gp130 include rapid activation of the Janus kinase/Stat pathway as well as activation of ERK1/2 and phosphatidylinositol 3-kinase (22, 23). In the F10.9 melanoma cells, IL6RIL6 acted identically to what we reported for the IL6 and sIL-6R combination (30) by strongly inducing Stat1 and Stat3 phosphorylation, the Stat3 activation persisting for more than 24 h. We also detected a rapid and transient activation of ERK1/2 that was inhibited by PD98059 (data not shown). PD98059 is known to stimulate melanogenesis (33), and we confirmed in F10.9 cells that it increases the activity of the /H11002/H11001/MITF promoter reporter as well as melanin. However, this mitogen-activated protein kinase inhibitor did not prevent the inhibitory effects of IL6RIL6 on melanogenesis and on the MITF promoter activity (data not shown).

The role of Stat3 was analyzed using dominant negative (DN) mutants of Stat3 (36) that we created in the mouse Stat3/APRF cDNA (51) by mutating the tyrosine phosphorylation site (Y705F), the serine phosphorylation site (S727A), and the DNA binding site (E434A/E435A). Pools of F10.9 cells permanently transformed with these Stat3DN plasmids were transfected by either a reporter gene driven by repeats of the STAT-RE or by the /H11002/MITF promoter reporter construct. The three Stat3DN mutants impaire the IL6RIL6-induced STAT-RE response to similar extents (Fig. 7A). However, the inhibition of the MITF promoter by IL6RIL6 was virtually abolished only by the Stat3 Y705F mutant, whereas the other Stat3DN mutant had a partial effect (Fig. 7B). The ability of the Stat3 Y705F DN mutant to block the action of IL6RIL6 was confirmed by analysis of the Mitf and Pax3 mRNA levels (Fig. 7C). The stably transformed clones in which this Stat3DN gene product was expressed (A2, A8, A9, A10) showed no or weak reduction in either Mitf or Pax3 mRNAs when treated by IL6RIL6, whereas in the transformed clones showing a low expression of Stat3DN (A4, A7), Mitf and Pax3 decrease as in the control pool B2. Immunoblots of total cell extracts showed also that the loss of the Pax3 protein caused by IL6RIL6 was abolished by the Stat3 Y705F DN-mutant, whereas the other two DN-mutants were partially effective (Fig. 7D).

We also followed the morphological change induced by IL6RIL6 in the F10.9 cells by transfection of the Stat3DN mutant plasmids that also co-expressed the green fluorescent protein EGFP. The fluorescent cells expressing Stat3 Y705F did not show the typical elongation of the F10.9 cells (such as seen in Fig. 1C), whereas elongation occurred with the other Stat3DN mutants (not shown). Hence, Stat3 tyrosine phosphorylation appears critical for the loss of Pax3, the ensuing loss of melanocytic phenotype, and transdifferentiation, observed in the melanoma cells treated with the gp130 activator IL6RIL6.

DISCUSSION

Treatment of B16/F10.9 melanoma cells with the IL6RIL6 chimera activates the IL-6 family receptor gp130 signaling. This causes a loss of melanogenesis and tyrosinase activity as well as loss of expression of Mitf, which is a key regulator of melanocytic differentiation and survival (3, 4). The melanoma cells stop growing (26) and acquire a new phenotype character-
ized by striking elongation of the cells and induction of glial fibrillary acidic protein. This intermediate filament protein is rarely observed in malignant melanoma (15, 18) and is typical of glial cells, where it is induced through gp130 signaling (52). Moreover, we report elsewhere that gene markers of myelinating Schwann cells are induced in the course of F10.9 cells treatment by IL6RIL6 (46).2 Interestingly, when treated with forskolin, the same melanoma cells can instead undergo melanocytic differentiation with increased melanogenesis, increased tyrosinase, and higher Mitf expression. This bi-directional differentiation suggests that the F10.9 cells recapitulate the bipotentiality of melanocyte/Schwann cell precursors derived from the vertebrate neural crest cells, which show developmental acquisition of markers specific to either cell lineage (53, 54). The effects of IL6RIL6 on the F10.9 cells may provide an interesting model to study the mechanisms in such transdifferentiation.

**Fig. 5.** Ectopic expression of Pax3, but not Sox10, overrides down-regulation of MITF promoter by IL6RIL6. F10.9 cells were co-transfected with the MITF-luciferase (−381/+99) construct (0.5 μg/well), pSV2-β-galactosidase (0.5 μg/well). Total amount of expression vector was maintained at 1 μg/well by complementing pcPax3 (A and C) or pcSox10 (B and D) with pcDNA3. Each assay was done in duplicate. In A and B, we show the luciferase activity normalized on β-galactosidase from one representative experiment. In C and D, we show the ratio of luciferase values in non-treated (NT) samples over IL6RIL6-treated samples (average of four experiments performed in duplicate). E, immunoblot of nuclear proteins from F10.9 cells transfected with 2.0 μg/well of pcPax3 or pcSox10 treated (+) with IL6RIL6 or kept non-treated (−). Signals obtained with anti-Pax3 or anti-Sox10 antibodies are shown.

**Table IV**

| Expression vectors | IL6RIL6 | MITF-luc | Fold reduction | Pax3 mut1 | Fold reduction | Pax3 mut1,2 | Fold reduction | CRE mut | Fold reduction |
|--------------------|---------|----------|----------------|-----------|----------------|-------------|----------------|---------|----------------|
| pcDNA3             | −       | 100      |                | 7         | 3              | 7           | 1              | 7       | 1.0            |
| pcPax3             | +       | 112      | 3.3            | 14        | 1.9            | 3           | 0.8            | 16      | 0.9            |
| pcSox10            | −       | 116      | 2.1            | 15        | 1.5            | 8           | 1.6            | 13      | 3.3            |
| PcSox10+           | +       | 440      | 3.9            | 68        | 1.5            | 8           | 1.6            | 13      | 3.3            |
| pcPax3             | +       | 90       | 4.8            | 28        | 2.4            | 8           | 1.8            | 34      | 5.5            |

Pax3 and Sox10 synergism on the MITF promoter is affected by IL6RIL6

Native −381/+99 or modified MITF-Luc constructs were transfected in F10.9 cells (0.7 μg/well) together with expression vectors pcPax3 or pcSox10 (0.35 μg/well, completed to 0.8 μg/well by pcDNA3). The Pax3 binding site −260/−244 is mutated in Pax mut1 and as well in Pax mut1,2, where in addition, the putative Pax3 site −40/−26 (9) is deleted (see Fig. 3). CRE mut is mutated on the CRE site (−147/−141). Two experiments were done in duplicate with similar results. Normalized luciferase activity is shown together with fold reduction by IL6RIL6. S.D. is less than 15%.
Expression vector amounts were maintained at 1/\mu g/well, pSV2-\beta-galactosidase (0.5 \mu g/well), and different amounts of expression vectors pPax3 (P), pCterm Sox10 (S), or both. Expression vector amounts were maintained at 1 \mu g/well. NT, non-treated; Cont., control.

Down-regulation of the Melanocytic Program of Gene Expression—Treatment of the F10.9 melanoma cells by IL6RIL6 triggers repression of a hierarchy of transcription factors, which controls tyrosinase and melanogenesis. The >90% reduction in tyrosinase enzymatic activity per unit of total protein could be traced to a loss of tyrosinase mRNA and inactivation of the tyrosinase promoter in the IL6RIL6-treated cells. The reduced activity of M-box-driven reporter gene suggested that the MITF transcription factor was responsible for the decreased tyrosinase. Indeed, we observed a profound decrease in Mitf mRNA after IL6RIL6 addition to the cells, and when Mitf was ectopically expressed in these cells the inhibition of the tyrosinase promoter was not seen any more. Assay of reporter genes regulated by human MITF 5'-flanking sequences established that IL6RIL6 suppresses the transcriptional activity of the MITF gene promoter. Because a number of transcription factors regulate the 300-base pair proximal promoter domain of the MITF gene, which is highly conserved between mouse and human genes (55), we attempted to identify the target of IL6RIL6 action on this promoter. Analysis of the function of MITF DNA sequence after deletions or mutations indicated that the Pax3 binding site located at -260/-262 element just upstream of the Pax3 site in the MITF promoter.

The Pax3-Sox10 synergism has been observed (9) even in MITF constructs deleted of the Sox10 site -268/-262, close to the first Pax3 site, suggesting that Sox10 may stimulate Pax3 without a specific Sox10 binding site. A likely explanation is that Sox10 is recruited to the promoter through Pax3. We similarly found that a reporter gene driven by eight repeats of the first Pax3 site of the MITF promoter (PaxMS) is stimulated not only by Pax3 but also by Sox10 and synergistically by both. IL6RIL6 treatment reduced the transcriptional activity of the Pax3 repeats in the presence of Sox10 much more than with ectopic Pax3, strengthening the conclusion that the loss of the Pax3 protein and transcriptional activity is the prominent feature of the action of IL6RIL6 on the melanoma cells. Again, the synergism of Pax3 and Sox10 on the PaxMS synthetic promoter was lost in the IL6RIL6-treated cells.

The variations in the level of Sox10 protein in the F10.9 cells treated by IL6RIL6 indicate an early moderate decrease followed by an increase. Because Pax3 is steadily decreased, the ratio of Sox10 over Pax3 rises to high values after 24 h. The changes in Pax3 and Sox10 concentration and activity induced by the gp130 activator IL6RIL6 may be of broad interest in view of the roles of these factors in melanocytic and neuro-glial development. Pax3 functions as a gene essential for the melanocytic cell lineage. Heterozygous Pax3 gene mutations or deletions in splotch mice (59) as well as in the human Waardenburg syndrome types 1 and 3 (60) result in pigmentary and auditory defects, since melanocytes play a role in hearing (61). Mutations in MITF cause Waardenburg syndrome 2, whereas mutations in Sox10 are related to Waardenburg syndrome 4-Hirschsprung disease (62). Homozygous inactivating mutations of Sox10 prevent the development of Mitf-expressing melanocytic cell lineage. Heterozygous Pax3 gene mutations or deletions in splotch mice (59) as well as in the human Waardenburg syndrome types 1 and 3 (60) result in pigmentary and auditory defects, since melanocytes play a role in hearing (61). Mutations in MITF cause Waardenburg syndrome 2, whereas mutations in Sox10 are related to Waardenburg syndrome 4-Hirschsprung disease (62). Homozygous inactivating mutations of Sox10 prevent the development of Mitf-expressing melanoblasts (9, 63). Mutations in Sox10 in Dom mice affect melanocytic lineages as well as Schwann cell development (64). Sox10 is expressed throughout Schwann cell development from neural crest progenitors (44), whereas Pax3 is only expressed in early and premelinyling Schwann cell precursors but has to disappear for the terminal differentiation in myelinating cells expressing myelin protein genes (65). We have reported that treatment of embryonic Schwann cell cultures with IL6RIL6 leads to a strong decrease in Pax3 and induction of myelin gene products P0 and myelin basic protein (66). Moreover, in the F10.9 melanoma cells treated by IL6RIL6, we found that the
decrease in Pax3 and increase in Sox10 are associated with the induction of myelin Po and myelin basic protein mRNAs and activation of the promoters of these genes. 

Role of Stat3 and Other Signaling Pathways—IL6RIL6 binds to gp130 with higher affinity than the IL6 and soluble IL6R combination (67), and we know that its action on F10.9 cells can be inhibited by anti-gp130 antibodies. 

In F10.9 cells, the IL6 plus soluble IL6R combination activates Stat1 and Stat3 to bind palindromic enhancers (pIRE or STAT-RE) and to stimulate transcription of the tumor suppressor IRF-1 and p21/Waf-1 genes (30). Inhibition of Pax3 and Mitf by IL6RIL6 was severely reduced in F10.9 melanoma cells overexpressing a Stat3a mutant where the Tyr residue 705, normally phosphorylated rapidly in response to cytokine activation, was mutated. The same mutant also prevented the cell elongation, characterizing the phenotypic transition in response to IL6RIL6. Although mutations inactivating the Stat3 DNA binding site (68) or preventing Ser-727 phosphorylation were as efficient as Tyr-705 mutation in reducing STAT-RE-dependent transcription, they affected less IL6RIL6 effects on MITF transcription, Pax3 expression, and cell shape. This suggests that Stat3 may affect Pax3 and cell shape by modulating signaling networks rather than by activating STAT target genes. STAT factors are known to associate with accessory proteins in large complexes in the cytoplasm (69), and STAT signaling functions are regulated by homo- and heterodimerization with STAT-family or non-STAT factors (28). Stat3 was shown recently to interact with a small GTPase, Rac-1, which modulates Stat3 activation (70–72).

Small GTPases play important roles in regulating cytoskeletal changes and gene transcription. Activation of Rac-1 or inhibition of Rho-B in melanocytes mediates dendrite formation and tyrosinase transcription in response to α-MSH (40, 73). It could be speculated that interaction of Tyr-phosphorylated Stat3 with small GTPases plays a role in the changes of cell shape and the loss of melanogenesis in the F10.9 cells.

Pax3 and Melanoma Tumor Growth—Pax3 is potentially tumorigenic (74), and advanced melanoma seem to express more Pax3 than benign melanoma or melanocytes (75). Anti-sense Pax3 oligonucleotides induced apoptosis in melanoma tumors or in melanoma lines such as A375 (75), a phenomenon we have also observed in B16/F10.9 cells (not shown). It is therefore of interest that a chimeric cytokine such as IL6RIL6
markedly reduces the level of Pax3 protein in F10.9 cells, although the cells do not die but, rather, transdifferentiate to a glial/Schwann cell phenotype. The down-regulation of Pax3 by IL6RIL6 may be implicated in the growth arrest of F10.9 cells in addition to the reported induction of tumor suppressors IRF-1 and p21/waf1 in these cells treated with IL6 and soluble IL6R (30). We have observed the down-regulation of Pax3 also in other melanoma such as K1735 cells treated by IL6RIL6 (not shown). Our preliminary data indicate that the main mechanism of the down-regulation of Pax3 mRNA in IL6RIL6-treated cells is post-transcriptional. Whether such inhibition of Pax3 may be found in various types of human melanoma tumors in response to gp130 stimulators remains to be explored.

Acknowledgments—We thank Dr. Benjamin Geiger and Dr. Roni Seger (Immunology Department, Weizmann Institute of Science), Dr. Robert Ballotti (INSERM U385, Faculte de Medecine, Nice), and Dr. Ehud Razin (Biochemistry Department, Hebrew University Medical School, Jerusalem) for fruitful discussions and for help. We are grateful to Dr. Akira (Osaka Medical School) for the gift of pBS-APRF. The excellent technical assistance of Perla Federman, Rosa Kaufman, Nili Nisim, Raya Zwan, and Zipora Marks is gratefully acknowledged. We thank Dr. Dalia Guria-Rotman for collaboration, in particular with the Stat3DNRN-transformed cells.

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