Induction of Melanocyte-specific Microphthalmia-associated Transcription Factor by Wnt-3a*

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Microphthalmia-associated transcription factor (Mitf) plays a critical role in the development of neural crest-derived melanocytes. Here, we show that exogenously added Wnt-3a protein, an intercellular signaling molecule, up-regulates the expression of endogenous melanocyte-specific Mitf (Mitf-M) mRNA in cultured melanocytes. The melanocyte-specific promoter of the human MITF gene (MITF-M promoter) contains a functional LEF-1-binding site, which is bound in vitro by LEF-1 and confers the preferential expression on a reporter gene in melanocytes and melanoma cells, as judged by the transient transfection assays. Moreover, the LEF-1-binding site is required for the transactivation of a reporter gene by LEF-1, β-catenin, or their combination. Exogenously added Wnt-3a protein also transactivates the MITF-M promoter via the LEF-1-binding site; this activation was abolished when a dominant-negative form of LEF-1 was coexpressed. These results suggest that Wnt-3a signaling recruits β-catenin and LEF-1 to the LEF-1-binding site of the MITF-M promoter. Therefore, the present study identifies Mitf-M/MITF-M as a direct target of Wnt signaling.

Microphthalmia-associated transcription factor (Mitf),1 encoded by the mouse Mitf locus, plays a critical role in the differentiation of various cell types, including neural crest-derived melanocytes, bone marrow-derived mast cells and osteoelasts, and optic cup-derived retinal pigment epithelium (RPE) (1–3). Mitf and its human counterpart, MITF, contain a basic helix-loop-helix leucine zipper structure, which is required for DNA binding and dimer formation. MITF consists of at least five isoforms with distinct amino termini, called MITF-A, -B, -C, -H, and -M (4–6). The amino termini of these isoforms are encoded by a separate first exons, and each exon 1 is under the control of a unique promoter (6). Among these isoforms, MITF-M/Mitf-M is exclusively expressed in melanocytes and melanoma cells of neural crest origin (4, 5, 7). In fact, the 5′-flanking region of the first exon, coding for the amino terminus of MITF-M, shows the melanocyte-specific promoter function (8), here referred to as the MITF-M promoter. In contrast, other MITF isoforms are widely expressed in many cell types (4, 5).

MITF-M/Mitf-M efficiently transactivates the melanogenesis enzyme genes, such as tyrosinase and tyrosinase-related protein-1, in cultured cells (9–14) and can convert a fibroblast cell line to the cells expressing tyrosinase and tyrosinase-related protein-1 (15). The mutations in the MITF/Mitf gene were found in patients with auditory pigmentary syndromes such as Waardenburg syndrome type 2 (16–18), as well as in many Mitf mutant mice (2). These affected individuals mainly exhibit hypopigmentation and hearing impairment, caused by the lack of pigment cells in the skin and inner ear. Moreover, the MITF-M promoter is up-regulated by FAX3 (19), a transcription factor with a paired-homeodomain, in which the gene is responsible for Waardenburg syndrome types 1 and 3 (17). Moreover, the essential requirement of Mitf-M in melanocyte development was verified by the molecular lesion of the black-eyed white Mitf<sup>−/−</sup> mouse (20), which are characterized by a completely white coat color, deafness, and normally pigmented RPE (21). In Mitf<sup>−/−</sup> mice, the insertion of an L1 retrotransposable element in intron 3 lead to complete repression of Mitf-M mRNA expression and to a reduction of Mitf-A and Mitf-H mRNAs expression (20). Taken together, these results indicate that MITF-M/Mitf-M is a key regulator of the melanocyte development but is dispensable for RPE development. However, the mechanism of differentiation of neural crest cells toward melanocytes is not well understood.

Wnt proteins, which are secreted cysteine-rich glycoproteins, have been established as developmentally important signaling molecules (22). Particularly, Wnt-1 and Wnt-3a are required for the expansion of neural crest precursors (23, 24) and for determining the fate of neural crest cells during early development (25). In fact, targeted disruption of the Wnt-1 and Wnt-3a genes in the mouse causes deficiency of neural crest derivatives, including melanocytes (24). On the other hand, mutant mice lacking Wnt-1 or Wnt-3a show no noticeable deficiency of neural crest derivatives from the dorsal neural tube (26, 27). These results suggest a redundant role for Wnt-1 and Wnt-3a signaling in the differentiation of neural crest precursors. Wnt-3a is expressed in pluripotent ectoderm cells of the primitive streak during gastrulation (27). The onset of Wnt-3a expression is detected at embryonic day 7.5 (27), which precedes the onset of Mitf expression in neural crest cells (9.5–10.5 days) (28). These results suggest that Wnt-3a is a good candidate for regulating the differentiation of neural crest cells toward melanocytes. The signals evoked by Wnt proteins lead to intracel-
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lular accumulation of β-catenin, a key downstream component of the Wnt signaling pathway (22, 29). β-Catenin then activates the target genes through interaction with a member of the LEF-1/TCF transcription factors, containing a high mobility group domain. Thus, LEF-1/TCF transcription factors mediate a nuclear response to Wnt signals.

Here, we show that exogenously added Wnt-3a protein induces endogenous Mitf-M mRNA in cultured melanocytes. In addition, we identify the functional LEF-1-binding site in the MITF-M promoter and provide evidence that Wnt-3a signaling recruits β-catenin and LEF-1 to the MITF-M promoter, which leads to increased transcription from the MITF-M promoter. Therefore, the present study shows a direct link between Wnt signaling and Mitf-MMITF-M expression.

EXPERIMENTAL PROCEDURES

Wnt-3a Conditioned Medium—The mouse fibroblast L cells, constitutively expressing mouse Wnt-3a cDNA, were seeded at a density of 1 × 10^6 in a 94-mm dish containing a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 supplemented with 10% fetal calf serum. After 3 days of culture, cells were refed with fresh medium, incubated for 1 more day, and the cultured media were collected as conditioned media, referred to as Wnt3a/L-CM. Wnt3a/L-CM was estimated to contain about 400 ng/ml of Wnt-3a protein (30). Conditioned media, neo/L-CM, were also prepared from the cultures of control L cells that were stably transfected with a vector plasmid as described previously (30).

Northern Blot Analysis—Mela-n-a murine-immortalized melanocytes, a gift from D. C. Bennett (31), were grown in minimum essential medium supplemented with 10% fetal calf serum and 200 nM phorbol 12-myristate 13-acetate. Melan-a cells, maintained in a 3.5-cm dish containing 1 ml of the medium, were treated with Wnt3a/L-CM or neo/L-CM for 24 h. The final concentration of Wnt-3a was about 40 ng/ml. Total RNA was prepared from the treated Melan-a cells of two dishes and subjected to Northern blot analysis as described previously (15). The Clal/EcoRI DNA fragment of human MITF cDNA (32) and glyceraldehyde 3-phosphate dehydrogenase cDNA (positions 601–1052) (33) were labeled with [α-32P]dCTP using a BcaBEST labeling kit (Takara) and were used as hybridization probes.

Plasmid Preparation—A wild-type reporter plasmid, pGL3-MITF/M, was constructed as follows. The BamHI/XhoI fragment containing the 2.2-kilobase pair human MITF promoter and provide evidence that Wnt-3a signaling activates the target genes through the interaction of β-catenin and a member of the LEF-1/TCF transcription factors. The consensus DNA sequence recognized by LEF-1/TCF transcription factors is CTTTGA/TA/T (29), and the consensus sequence motif is CTTGTAT (Fig. 2B). The same sequence motif is conserved at a similar position in the mouse MTET-M promoter (15). The same sequence motif is conserved at a similar position in the mouse MITF-M promoter (Fig. 2A). The same sequence motif is conserved at a similar position in the mouse Mitf-M/MITF-M promoter is activated in many melanoma cells due to β-catenin mutation (36). In fact, the expression level of pGL3-MITF-M/MITF-M was decreased in melanoma cells due to β-catenin mutation (36). In fact, the expression level of pGL3-MITF-M/MITF-M was decreased in melanoma cells due to β-catenin mutation (36).

RESULTS AND DISCUSSION

To assess the hypothesis that Wnt signaling regulates the expression of Mitf-M/MITF-M, we analyzed the effect of Wnt-3a protein on Mitf-M mRNA expression in cultured melanocytes. Accordingly, Melan-a immortalized melanocytes were treated with Wnt3a/L-CM containing Wnt-3a protein or neo/L-CM for 24 h (Fig. 1). The treatment with Wnt3a/L-CM increased Mitf-M mRNA by 24 h (lane 1), whereas the treatment with neo/L-CM did not (lane 2). Thus, Wnt-3a induces the expression of endogenous Mitf-M mRNA, supporting the notion that Wnt signaling is involved in melanocyte differentiation.

Wnt signaling activates the target genes through the interaction of β-catenin and a member of the LEF-1/TCF transcription factors. The consensus DNA sequence recognized by LEF-1/TCF transcription factors is CTTTGA/TA/T (29), and the MITF-M promoter contains a putative LEF-1/TCF-binding site, CTTTGA/T (positions −199 to −193), which agrees with the consensus sequence (Fig. 2A). The same sequence motif is conserved at a similar position in the mouse Mitf-M/MITF-M promoter (2). To assess the function of the putative LEF-1/TCF-binding site in the MITF-M promoter, we compared the expression levels of pGL3-MITF-M/MITF-M in HMV-II human melanoma cells with those of pGL3-MITF-M/MITF-M, containing a deletion of the LEF-1/TCF site, CTTTGA/T (Fig. 2B). These base changes were expected to reduce the MITF-M promoter activity, because the Wnt signaling pathway is activated in many melanoma cells due to β-catenin mutation (36). In fact, the expression level of pGL3-MITF-M/MITF-M

CpGL3-MITF/M. The data shown are the means to the normalized luciferase activity in HeLa cells transfected with pGL3-MITF/M(m195). Relative luciferase activity is shown as the ratio to the normalized luciferase activity obtained with pGL3-MITF/M. The data shown are the means ± S.D. of three independent experiments.

MHV-II melanoma and HeLa cervical cancer cells were transfected with pGL3-MITF/M or pGL3-MITF/M(m195), containing the altered LEF-1/TCF-binding site. These competitors were added to the reaction mixture at a 1,000-fold molar excess over the input probe. The upper arrow indicates the LEF-1/DNA complex. The reticulocyte lysate containing the translation products derived from pRc/CMV was also subjected to EMSA as a negative control (lane 4).

CTTTGAT motif is involved in MITF-M promoter activity in melanoma cells. The significant luciferase activity detected with pGL3-MITF/M(m195) in melanoma cells may be due to the presence of other melanocyte-specific enhancers present in the MITF-M promoter.

We then performed cotransfection assays in HeLa cervical cancer cells to test the possibility that the LEF-1/TCF family is involved in regulation of the MITF-M promoter (Fig. 2C). Expression of either LEF-1 or β-catenin significantly increased the luciferase activity under the control of the MITF-M promoter. The coexpression of β-catenin and LEF-1 synergistically increased the luciferase activity, which was higher than the degree of activation caused by LEF-1 or β-catenin. In contrast, the introduction of mutation at the putative LEF-1 site completely inhibited the increase in luciferase activity caused by LEF-1, β-catenin, or their combination (Fig. 2C). These results suggest that the CTTTGAT motif of the MITF-M promoter represents a functional LEF-1-binding site.

To confirm whether LEF-1 protein binds to the CTTTGAT motif of the MITF-M promoter, we carried out EMSA. The in vitro translation of LEF-1 mRNA was confirmed by autoradiography of 35S-labeled LEF-1 protein (data not shown). The synthetic LEF-1-binding site was specifically bound by the in vitro translated LEF-1 protein (Fig. 3, lane 1). The formation of this complex was inhibited by competitor oligonucleotide containing the CTTTGAT motif but not by the mutant oligonucleotide containing the CTTTGAT motif (lanes 2 and 3). Taken together, these results indicate that LEF-1 recognizes the CTTTGAT motif of the MITF-M promoter. These results are consistent in part with the recent report showing that the LEF-1/TCF-binding sites of the promoter region of the Nacre, a zebrafish homolog of MITF, is required for pigment cell-specific expression of a reporter gene in vivo (37).

Finally, we assessed the effects of Wnt-3a protein on the MITF-M promoter activity in Melan-a cells (Fig. 4). In this case, the expression levels of these two constructs were similarly lower in HeLa cervical cancer cells. These results suggest that the
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It is therefore conceivable that Wnt signals activate the MITF-M promoter by recruiting LEF-1 and β-catenin to the LEF-1-binding site. Thus, MITF-M/Mitf-M is a direct target gene of Wnt signaling in humans and mice.

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In summary, Wnt-3a protein induces Mitf-M mRNA expression in melanocytes and activates the MITF-M promoter by recruiting LEF-1 and β-catenin to the LEF-1-binding site. Thus, MITF-M/Mitf-M is a direct target gene of Wnt signaling in humans and mice.

A certain factor other than Wnt-3a in the conditioned medium is necessary and sufficient for the activation of the MITF-M promoter by about 2.5-fold. To confirm that this increase was to that obtained with pGL3-MITF/M alone. The data shown are the means ± S.D. of three independent experiments.

series of experiments, Melan-a immortalized melanocytes were chosen because this cell line was more sensitive to Wnt-3a treatment than HMV-II melanoma cells, as judged by transient expression assays with the test plasmid TOPFLASH, containing multiple LEF-1 responsive elements (data not shown). Such a difference in sensitivity to Wnt-3a suggests that a component(s) of the Wnt signaling pathway, such as β-catenin, may be constitutively activated in HMV-II melanoma cells.

Melan-a cells were transfected with pGL3-MITF/M or pGL3-MITF/M (m195) and then treated with either Wnt3a/L-CM or neo/L-CM. Wnt3a/L-CM increased the expression of pGL3-MITF/M by about 2.5-fold. To confirm that this increase was dependent on Wnt signaling, we cotransfected the expression plasmid of a dominant-negative form of LEF-1, pRc/CMV-DN-LEF-1. The dominant-negative LEF-1 lacks the amino-terminal β-catenin interaction domain and is expected to inhibit Wnt signaling. The observed activation by Wnt3a/L-CM was completely inhibited when the dominant-negative LEF-1 was coexpressed. Furthermore, the expression level of pGL3-MITF/M (m195) was lower than that of a wild-type construct, pGL3-MITF/M, and was not significantly increased by treatment with Wnt3a/L-CM. These results indicate that the LEF-1 site is necessary and sufficient for the activation of the MITF-M promoter by Wnt-3a signaling. The data all support the interpretation of a direct effect by Wnt-3a, but there is a possibility that a certain factor other than Wnt-3a in the conditioned medium from the Wnt3a transfectants could contribute to the LEF-1-dependent activation. Further study with Wnt-3a neutralizing antibodies will be required to address this issue.

The MITF-M promoter is functionally exclusive in melanocyte-lineage cells (6, 8) and is up-regulated via the separate cis-acting elements by Pax3 (19) and by a-melanocyte-stimulating hormone signaling (38) (see Fig. 2A). Here, we provide evidence that Wnt-3a signal activates the MITF-M promoter through the LEF-1 binding site. Thus, multiple signals appear to converge on the MITF-M promoter, leading to the up-regulation of MITF-M expression, a key regulator for the melanogenesis enzyme genes. The expression levels of Pax3 mRNA were reduced in the double knock-out mouse of the Wnt-1 and Wnt-3a genes (24). It is therefore conceivable that Wnt signals may also up-regulate Mitf-M/MITF-M expression through Pax3.

In summary, Wnt-3a protein induces Mitf-M mRNA expression in melanocytes and activates the MITF-M promoter by recruiting LEF-1 and β-catenin to the LEF-1 binding site. Thus, MITF-M/Mitf-M is a direct target gene of Wnt signaling in humans and mice.
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