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Functional Difference of the SOX10 Mutant Proteins Responsible for the Phenotypic Variability in Auditory-Pigmentary Disorders

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Waardenburg syndrome (WS) is an inherited disorder, characterized by auditory-pigmentary abnormalities. SOX10 transcription factor and endothelin receptor type B (EDNRB) are responsible for WS type 4 (WS4), which also exhibits megacolon, while microphthalmia-associated transcription factor (MITF) is responsible for WS2, which is not associated with megacolon. Here, we investigated the functions of SOX10 mutant proteins using the target promoters, *EDNRB* and *MITF*. The SOX10 mutations chosen were E189X, Q377X, and 482ins6, which are associated with WS4, and S135T that is associated with Yemenite deaf-blind hypopigmentation syndrome (YDBS), which does not manifest megacolon. These SOX10 mutant proteins showed impaired transactivation activity on the *MITF* promoter. In contrast, E189X and Q377X proteins, each of which lacks its C-terminal portion, activated the *EDNRB* promoter, whereas no activation was detected with the SOX10 proteins mutated at the DNA-binding domain, 482ins6 and S135T. However, unlike 482ins6 protein, S135T protein synergistically activated *EDNRB* promoter with a transcription factor Sp1, indicating that Sp1 could compensate the impaired function of a SOX10 mutant protein. We suggest that the variability in transactivation ability of SOX10 mutant proteins may account for the different phenotypes between WS4 and YDBS and that Sp1 is a potential modifier gene of WS4.

Key words: auditory-pigmentary disorder, EDNRB, MITF, SOX10, Sp1.

Abbreviations: EDN, endothelin; EDNRB, endothelin receptor type B; GST, glutathione S-transferase; HMG, high mobility group; MITF, microphthalmia-associated transcription factor; PAX3, paired box gene 3; SOX10, Sry-box 10; Sp1, specificity protein 1; WS, Waardenburg syndrome; YDBS, Yemenite deaf-blind hypopigmentation syndrome.

Transcription factors play critical roles in regulatory networks of many developmental pathways, cell growth and differentiation, and mutations in the genes encoding the transcription factors are associated with various human disorders that are frequently inherited in a dominant manner (*1*). Waardenburg syndrome (WS) is such an example and is characterized by varying combinations of sensorineural hearing loss, heterochromia iridis, and patchy abnormal pigmentation of the hair and skin (*2*). WS is caused by the absence of neural crest–derived melanocytes, and is classified into four types, WS1 to WS4, depending on the presence or absence of additional symptoms (*3–12*). There are at least six genes responsible for WS, including *microphthalmia-associated transcription factor* (*MITF*), *PAX3, SOX10*, and *endothelin receptor type B* (*EDNRB*) (*3–12*). The *MITF* gene is responsible for WS2 (*13–16*), which shows no additional symptoms distinguished from WS1, 3, and 4. PAX3 is a transcription factor, which is responsible for WS1 and WS3 (*6*). SOX10 is a transcription factor, which contains the high-mobility group (HMG) box as the DNA-binding domain (*17*). The mutations of the *SOX10* gene are associated with some cases of WS4 (*3–5*) and Yemenite deaf-blind hypopigmentation syndrome (YDBS) (*18*). WS4, referred to as Hirschsprung's disease type 2 or Shaa-Waardenburg syndrome, is characterized by the presence of aganglionic megacolon. YDBS manifests cutaneous pigmentation abnormalities, early onset of hearing loss, microcornea and colobomata (*19*), but its mild form is not associated with ocular defects (*18*). The mutation of SOX10 was found in the mild form of YDBS (*18*). The SOX10-associated WS4 and YDBS exhibit a marked phenotypic difference; for example, aganglionic megacolon is associated with WS4, but not with YDBS.

Several lines of evidence have suggested the functional relationships between these WS-related transcriptional factors. MITF expression is regulated by PAX3 and SOX10 (*13–16, 20, 21*). Recently, we have shown that SOX10, alone or in combination with a transcription factor Sp1, transactivates the *EDNRB* promoter in human melanocyte-lineage cells (*22*). EDNRB belongs to a superfamily of G protein-coupled receptors (*23*). The EDNRB-mediated signaling is involved in the development of the melanocytes and enteric nervous precursor cells (*24, 25*). Moreover, the *EDNRB* gene is responsible for WS4 (*10, 11*), and the *EDNRB*-associated WS4 is inherited in a dominant (*11*) or recessive (*10*) mode. On the other hand, SOX10 transactivates the melanocyte-specific promoter of the *MITF* gene, termed *MITF-M* promoter (*13–16, 20, 21*). MITF consists of at least nine isoforms with distinct first exons (*7, 8, 26–33*). Among these isoforms, MITF-M is exclusively expressed in melanocytes of neural crest

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origin and melanoma cells (*27, 34*). These results suggest that SOX10 mutant proteins may differentially influence the *EDNRB* or *MITF-M* promoter activity, which might account for the phenotypic difference between WS and YDBS.

Here we have identified the different transactivation ability of each SOX10 mutant protein on the *EDNRB* and *MITF-M* promoters, which could account for the phenotypic differences between WS4 and YDBS. Moreover, we provide evidence that Sp1 rescues the impaired function of SOX10 S135T, associated with YDBS, in the activation of the *EDNRB* promoter. Thus, the impaired function of SOX10 could be compensated for in part by Sp1. The functional analysis of the mutant proteins of WS-related genes will help us understand the relationship between the genetic disorders and the phenotypes.

MATERIALS AND METHODS

Cell Culture—HeLa human uterine cervical cancer cells and COS-7 monkey kidney cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2/95% room air. HMV-II human melanoma cells were obtained from RIKEN Cell Bank and cultured in nutrient mixture Ham's F12 medium containing 10% FBS at 37°C under 5% CO_2/95% room air.

Plasmids Construction—Human SOX10 expression vectors, pcDNA3-SOX10 and p3XFLAG-SOX10, and a human MITF expression vector were described previously (*16, 22, 34, 35*). A human Sp1 expression vector, pCIneo-Sp1, was a gift from Dr. Kojima (The Institute of Physical and Chemical Research, Tsukuba Life Science Center, Tsukuba, Ibaraki, Japan) (*36*). The human PAX3C cDNA (*37*), corresponding to the conventional mouse Pax3 isolated by Goulding, M.D. *et al.* (*38*), was generated by reverse-transcriptase polymerase chain reaction (PCR) from RNA of normal human epidermal melanocyte (KURABO, Osaka, Japan), and was inserted into the TA cloning plasmid pGEM-Teasy (Promega), yielding T-PAX3C. The fragment of PAX3C cDNA was inserted into the expression plasmid pcDNA3, yielding the pcDNA3-PAX3. A reporter construct of human *MITF-M* promoter, pGL3-MITF/M, and the human *EDNRB* promoter, pGL3-E (-3022), were described previously (*16, 22, 28, 39*). To construct the SOX10 mutant expression vectors, base changes were introduced into p3XFLAG-SOX10 using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instruction. In the case of SOX10 E189X mutant protein (E189X), glutamate (GAG) at position 189 of SOX10 was changed into the terminal codon (tAG) so that the truncated protein consists of the amino-terminal 188 residues. This mutant protein has the DNA-binding domain but lacks the transactivation domain. SOX10 Q377X mutant protein (Q377X), in which glutamine (CAG) at position 377 of SOX10 was changed to the terminal codon (tAG), lacks the C-terminal transactivation domain of 89 residues. SOX10 482ins6 mutant protein (482ins6) carries an insertion of 6 nucleotides (gctccg) between positions 482 and 483, resulting in the addition of a leucine residue and an arginine residue in the HMG box with the open reading-frame intact. However, the 482ins6 mutant protein does not have any DNA binding activity (*5*). Furthermore, SOX10 S135T protein (S135T), which carries threonine (AGC) at position 135 instead of serine (AcC), has no DNA binding activity (*18*).

Transient Transfection Assays—The activities of the human *EDNRB* and *MITF-M* promoters were assessed by transient expression of the firefly luciferase gene in HeLa or HMV-II cells, as described previously (*22*). Cells used were cultured for 24 h after plating in 12-well dishes and then transfected with each reporter plasmid (120 ng), pRL-TK (20 ng), and wild-type SOX10 cDNA (pcDNA3-SOX10 or p3XFLAG-SOX10) (50 ng), each SOX10 mutant cDNA (p3XFLAG-SOX10 mutants) (50 ng), Sp1 cDNA (50 ng), PAX3 cDNA (50 ng), or an empty expression vector, using the transfection reagent FuGENE 6 (Roche Diagnostics, Mannheim, Germany). For the analysis of the dominant-negative effect of each SOX10 mutant protein, HeLa cells were transfected with pGL3-E (-3022) (120 ng), pRL-TK (20 ng), p3XFLAG-SOX10 (50 ng), Sp1 cDNA (50 ng), and each of SOX10 mutant cDNAs (p3XFLAG-SOX10 mutants) (25 or 50 ng), or an empty expression vector. Total amount of plasmids transfected was 500 ng per well. An internal control pRL-TK contains the herpes simplex virus thymidine kinase promoter upstream of Renilla luciferase (Promega, Madison, WI, USA). The cells were harvested 24 h after transfection, and then luciferase activity was measured with Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized with each Renilla luciferase activity.

Western Blot Analysis—Whole cell extracts were prepared from the transiently transfected HeLa cells by the method of Schreiber, E. *et al.* (*40*) and subjected to western blot analysis using anti-FLAG antibody (Sigma-Aldrich Japan, Tokyo, Japan) or anti–α-tubulin antibody (NeoMarkers, Fremont, CA, USA).

Glutathione S-Transferase (GST)-Pull Down Assays—SOX10 and its mutant proteins were prepared as fusion proteins containing a FLAG epitope tag at its N-terminus. COS-7 cells (5×10^6) were transfected with 6 μg of SOX10 or each SOX10 mutant expression vector and harvested 24 h after transfection. The pGEX-3X-Sp1 was constructed by inserting the blunted *Xba*I/*Sma*I fragment of pCIneo-Sp1 into the SmaI site of pGEX-3X (Amersham Biosciences, Piscataway, NJ, USA). The GST-Sp1 fusion protein was prepared as previously described (*41*) and purified on glutathione (GSH)-Sepharose 4B resin (Amersham Biosciences), according to the manufacturer's instruction (Amersham Biosciences). COS-7 nuclear extracts were prepared by the method of Schreiber, E. *et al.* (*40*) and then preincubated with GSH-Sepharose 4B at room temperature for 1 h to reduce the endogenous GSH-Sepharose-4B binding. Nuclear extracts of COS-7 cells expressing SOX10 (10 μg of protein) were added to 50 μl of GST-Sp1 resin (5 μg of protein) suspension and diluted with cell lysis buffer (20 mM HEPES, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol-bis (2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 1 mM DTT, protease inhibitor cocktail (Sigma-Aldrich Japan), 20% glycerol, and 0.1% Triton-X) to adjust the salt concentration to about 50 mM NaCl. The sample was then incubated at room temperature for 1 h. The resin was washed five times with 500 μl of cell lysis buffer, and a final suspension of 20 μl was applied to SDS-PAGE. FLAG-tagged SOX10 was detected by western

blot analysis with anti-FLAG antibody (Sigma-Aldrich Japan).

Statistical Analysis—All data are mean ± SD of at least three independent experiments. Two-tailed Student's *t* test was used for comparison between the two groups. Differences between mean values were considered significant when $p < 0.01$.

RESULTS

Transactivation Ability of SOX10 and MITF-M on the EDNRB Promoter—*EDNRB* mRNA consists of at least four transcripts, named conventional *EDNRB, EDNRBΔ1, EDNRBΔ2*, and *EDNRBΔ3*, which are derived from three promoters of the human *EDNRB* gene (*42*). Among these transcripts, conventional *EDNRB* mRNA is most abundantly expressed in human melanocytes (*22*). To address a possibility whether MITF-M regulates the expression of the *EDNRB* gene, we analyzed the effect of MITF-M on the conventional *EDNRB* promoter, which is termed the *EDNRB* promoter in the subsequent text (Fig. 1A). We used the 3-kb length of the *EDNRB* promoter, because this region is able to confer the melanocyte-lineage specific expression on a reporter gene (*22*). MITF-M did not show any noticeable influence on the *EDNRB* promoter activity in HeLa cervical cancer cells (Fig. 1B, left panel) and HMV-II melanoma cells (Fig. 1B, right panel). In contrast, SOX10 transactivated the *EDNRB* promoter (about 3-fold) in HeLa cells and HMV-II cells, as reported previously (*22*). We next examined whether MITF-M influenced the *EDNRB* promoter when co-expressed with SOX10 and/or a transcription factor Sp1, as we have recently reported that SOX10 transactivates the *EDNRB* promoter in combination with Sp1 (*22*). Under the conditions used, MITF-M, in combination with Sp1 and/or SOX10, exerted no noticeable effects on the transactivation of the *EDNRB* promoter (Fig. 1C). Taken together, these results suggest that MITF-M may be dispensable for the melanocyte-specific expression of the *EDNRB* gene, although the possibility remains that MITF-M may be involved in the expression by interacting with other transcription factor.

Differential Effects of SOX10 Mutants on the MITF-M Promoter—We next examined the functions of SOX10 mutant proteins, each of which is associated with WS4 or YDBS, using the *MITF-M* promoter by the transient cotransfection assays (Fig. 2). In the present study, we focused on the *MITF-M* promoter, which plays a key role for the melanocyte-specific transcription of the *MITF* gene (*29, 34, 43*), and is regulated by SOX10 (Fig. 2A) (*13–16, 20, 21*). Wild-type SOX10 and SOX10 mutant proteins were tagged with FLAG, which was used for the confirmation of their expression levels. The mutant proteins chosen were SOX10 E189X, Q377X, and 482ins6, which are associated with WS4 (*3–5*), and SOX10 S135T, which is associated with YDBS (*18*) (Fig. 2B). The E189X and Q377X proteins represent truncated proteins with the intact HMG box, but they lack the C-terminal half (278 amino acids) and the C-terminal transactivation domain of 90 amino acids, respectively. The 482ins6 and S135T proteins have an in-frame insertion of two amino acids and a missense mutation at the HMG box, respectively, both of which lack the DNA-binding activity (*5, 18*). We initially

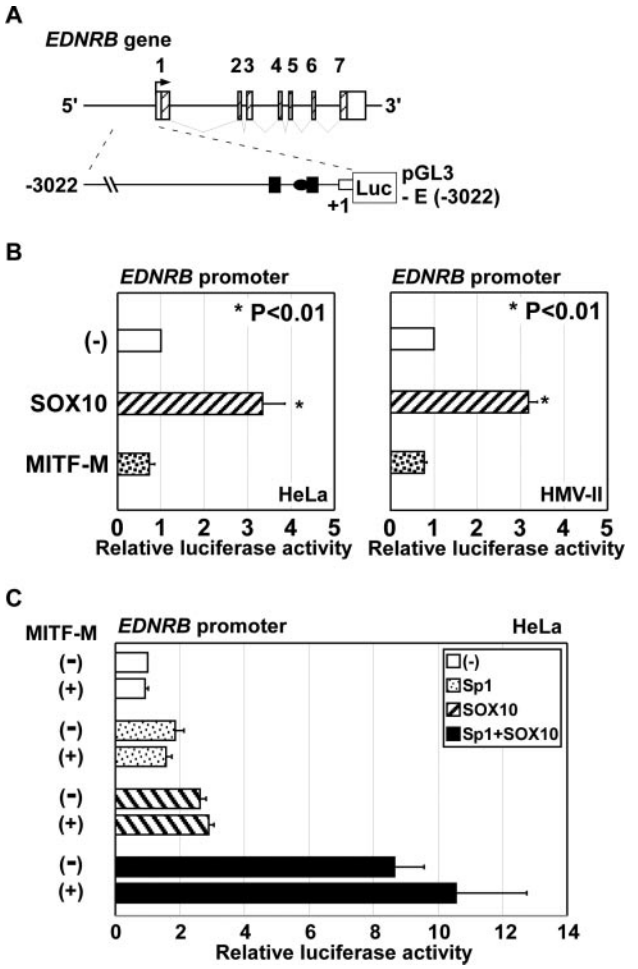

Fig. 1. **SOX10 transactivates the *EDNRB* promoter.** (A) The schematic representation of the *EDNRB* gene and the pGL3-E (-3022) reporter plasmid, containing the *EDNRB* promoter. The number shown on the *EDNRB* gene structure represents the number of exon. The exons are shown as the boxes on the *EDNRB* gene. The open portion is 5′- or 3′-untranslated region and the shadow portion is the coding region. The reporter construct used is shown below the *EDNRB* gene structure. The number shown at the 5′-end of the construct represents the position from the transcriptional initiation site (+1) of the promoter. The *cis*-elements responsible for SOX10 alone or both SOX10 and Sp1 are shown as the filled boxes or the filled circle, respectively. The open box upstream of the luciferase gene represents the 5′-untranslated region of *EDNRB* mRNA. (B) Effects of SOX10 or MITF-M on the *EDNRB* promoter. The panel shows the result of the transient transfection assay in HeLa cells (left) and HMV-II cells (right). The co-transfection with pGL3-E (-3022) and an empty vector (–), SOX10 expression vector (SOX10), or MITF-M expression vector (MITF-M) is shown as an open bar, a shadow bar, or a dotted bar, respectively. Relative luciferase activity is shown as the ratio to the normalized luciferase activity obtained with co-transfection of pGL3-E (-3002) and the empty vector. Data are mean ± SD of at least three independent experiments. The activity with * is significantly higher than the value obtained with co-transfection of pGL3-E (-3002) and empty vector, $p < 0.01$. (C) Effects of MITF-M on the transactivation of *EDNRB* promoter achieved by SOX10 and/or Sp1. HeLa cells were co-transfected with pGL3-E (-3022) and Sp1 expression vector, SOX10 expression vector, or a combination of SOX10 and Sp1 expression vectors, in the presence (+) or absence (–) of MITF-M expression vector. Relative luciferase activity is shown as the ratio to the normalized luciferase activity obtained with co-transfection of pGL3-E (-3002) and the empty vector. Data are mean ± SD of at least three independent experiments.

confirmed the similar expression levels of the SOX10 mutant proteins in the transfected HeLa cells (Fig. 2B).

SOX10 remarkably transactivated the *MITF-M* promoter, as reported previously (*13–16, 20, 21*). The SOX10 mutant proteins, E189X and 482ins6, did not transactivate the *MITF-M* promoter, although the SOX10 Q377X and S135T proteins retain the transactivation ability (Fig. 2C). It is noteworthy that S135T, but not 482ins6, shows the transactivation ability, despite that both S135T and 482ins6 proteins have the mutations in the HMG box. Because it has been reported that SOX10 synergistically transactivates the *MITF-M* promoter in combination with PAX3 (*14*), we examined the effect of the SOX10 mutant protein, Q377X, for the synergistic activation with PAX3 (Fig. 2D). In this context, it was reported that other SOX10 mutant proteins, used in this study, did not show the synergistic activation of the *MITF-M* promoter with PAX3 (*44*). In fact, SOX10 and PAX3 synergistically transactivated the *MITF-M* promoter, but the 482ins6 did not (Fig. 2D). Unexpectedly, Q377X showed the synergistic activation with PAX3, although the degree of coactivation was three-fold lower than that with wild-type SOX10. These results indicate the impaired functions of the four SOX10 mutants on the *MITF-M* promoter and their different biochemical consequences (Table 1). Moreover, Sp1, alone or in combination with SOX10 or each of SOX10

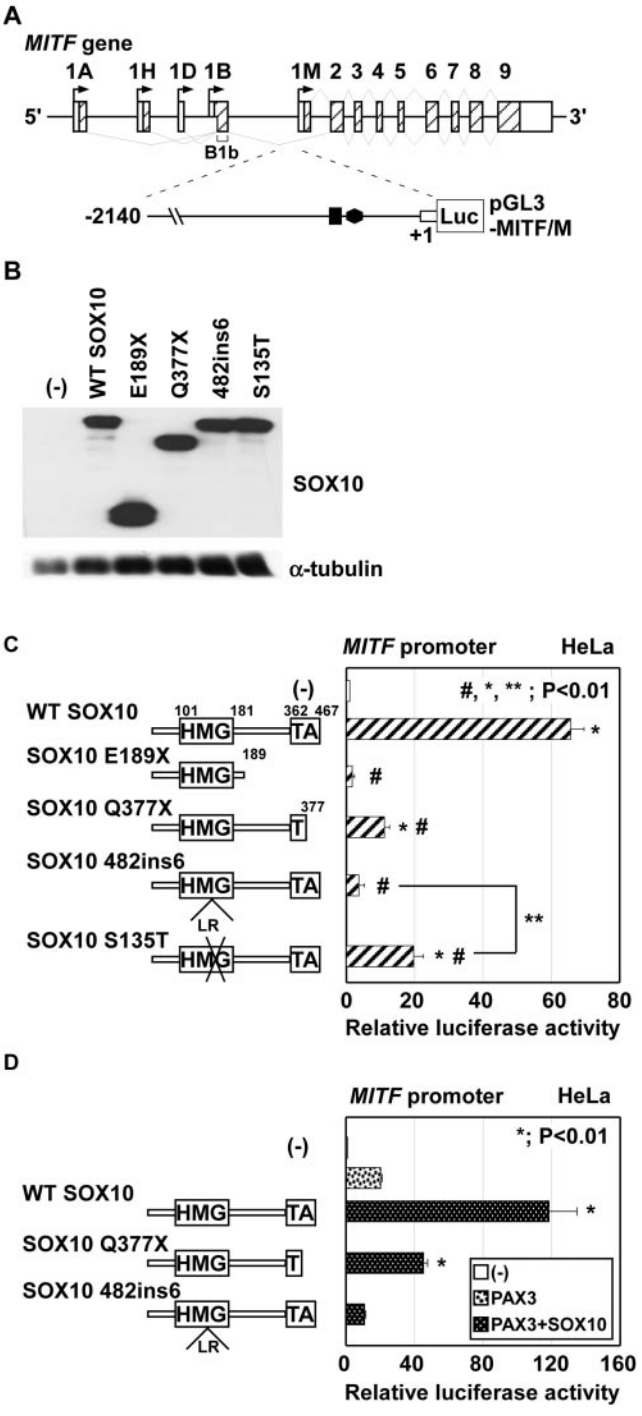

Fig. 2. **Impaired functions of the SOX10 mutant proteins on the *MITF-M* promoter.** (A) The schematic representation of the pGL3-MITF/M reporter plasmid, containing the *MITF-M* promoter. The exons are shown as the boxes. The number shown on the *MITF* gene structure represents the number of exon. The exon 1M is the melanocyte-specific exon 1. The exon 1A, 1H, 1D, or 1B is an alternative exon 1 of the *MITF* gene. The 3′-portion of exon 1B encodes domain B1b and is used as a common second exon, referred to as exon B1b, in the splicing of the primary transcripts initiated from exon 1A, 1H, and 1D. The open box is 5′- or 3′-untranslated region. The shadow box is the coding region. The reporter construct used is shown below the *MITF* gene structure. The number shown at the 5′-end of the construct represents the position from the transcriptional initiation site (+1) of the promoter. The representative SOX10-binding site or PAX3-binding site is shown as a filled box or a filled hexagon, respectively. The open box upstream of the luciferase gene (Luc) represents the 5′-untranslated region of *MITF-M* mRNA. (B) Expression of each SOX10 mutant protein in transfected HeLa cells. Each FLAG-tagged SOX10 mutant expression vector, shown on the top of the panel, was transiently expressed in HeLa cells. Each lane contained whole cell extracts (10 μg protein). The lane with (–) contains whole cell extracts of mock-transfected HeLa cells. Anti-FLAG antibody or anti–α-tubulin antibody was used for western blot analysis. (C) Effects of SOX10 mutant proteins on the *MITF-M* (*MITF*) promoter. The structure of each SOX10 mutant protein used was shown in the left. The number of amino acids is shown on the protein structure. The right panel shows the result of the transient transfection assay in HeLa cells. The co-transfection with pGL3-MITF/M and the empty vector (–) or each FLAG-tagged SOX10 expression vector is shown as an open bar or a shadow bar, respectively. Relative luciferase activity is shown as the ratio to the normalized luciferase activity obtained with co-transfection of pGL3-MITF/M and the empty vector. Data are mean ± SD of at least three independent experiments. The activity with * is significantly higher than the value obtained with the empty vector, $P < 0.01$. The activity of SOX10 S135T with ** is significantly higher than the value obtained with SOX10 482ins6 expression vector, $P < 0.01$. The activity with # is significantly lower than the value obtained with wild-type SOX10 expression vector, $p < 0.01$. Note that transactivation activity was undetected with SOX10 E189X or 482ins6 protein. (D) Effects of SOX10 mutant proteins on the synergistic activation of the *MITF* promoter with SOX10 and PAX3. The SOX10 mutant proteins used were Q377X and 482ins6. HeLa cells were co-transfected with pGL3-MITF/M, PAX3 expression vector, and FLAG-tagged wild type SOX10, Q377X or 482ins6 expression vector. Relative luciferase activity is shown as the ratio to the normalized luciferase activity obtained with co-transfection of pGL3-MITF/M and the empty vector shown as (–). PAX3 alone transactivated the *MITF* promoter (stippled box). Data are mean ± SD of at least three independent experiments. The activity with * is significantly higher than the value obtained with the PAX3 vector alone, $P < 0.01$.

Table 1. **Characteristics of the SOX10 mutant proteins.** The DNA binding activities, marked with "++", indicate that the mutant SOX10 protein has higher activity than that of wild-type SOX10. A symbol "+" or "–" indicates that the relevant activity of a mutant SOX10 protein is similar to or lower than that of wild-type SOX10, respectively. A symbol "ND" indicates that relevant activity is not detectable with the mutant SOX10 protein. The numbers shown represent the references about affected colon phenotype (*3, 4*), the DNA-binding activity (*5, 18, 46*), and the transactivation ability of *c-RET* promoter (*44, 51*).

		Affected colon phenotype	DNA binding activity	*in vitro*–binding with Sp1	Transactivation ability				*c-RET* promoter
					EDNRB promoter		*MITF* promtoter		
					Alone	Coactivation with Sp1	Alone	Coactivation with PAX3	
WS4	E189X	Short segment HSCR[3]	++[46]	+	+	–	ND	ND[44]	–[44]
	Q377X	Variable diagnosis ranging from hypoganglionosis to long segment HSCR[4]	++[46]	+	+	+	–	–	–[51]
	482ins6	Short segment HSCR[3]	–[5]	–	ND	ND	ND	ND[44]	–[44]
YDBS	S135T	Not detected	–[18]	+	ND	–	–	ND[44]	+[44]

++, higher than wild type; +, equivalent level to wild type;–, lower than wild type;ND; not detectable.

mutants, showed no noticeable effects on the degree of transactivation of the *MITF-M* promoter (data not shown), whereas Sp1 and SOX10 synergistically transactivated the *EDNRB* promoter (*22*).

Differential Effects of SOX10 Mutants on the EDNRB Promoter—We then analyzed the functions of each SOX10 mutant protein in the interaction with Sp1 and the transactivation of the *EDNRB* promoter. To explore the interaction between Sp1 and SOX10, we performed the pull-down assay using GST-fused Sp1 protein (GST-Sp1) and nuclear extracts of COS-7 cells expressing each SOX10 mutant protein (Fig. 3A). E189X, Q377X, or S135T protein bound GST-Sp1, as did wild-type SOX10. The interaction ability of 482ins6 protein was lower than that of wild-type SOX10. It should be noted that the interaction ability of 482ins6 protein was severely impaired, which is consistent with a predicted disruption of the HMG box structure in the 482ins6 protein (*5*) and with a previous report (*45*).

We next analyzed the effects of each mutant SOX10 protein on the *EDNRB* promoter activity (Fig. 3B). Either E189X or Q377X protein transactivated the *EDNRB* promoter, as did wild-type SOX10, whereas both mutant proteins showed impaired function on the *MITF-M* promoter (see Fig. 2B). Moreover, Q377X protein transactivated the *EDNRB* promoter in combination with Sp1, whereas E189X protein did not. In contrast, 482ins6 or S135T protein alone was unable to transactivate the *EDNRB* promoter. However, S135T protein, but not 482ins6 protein, showed synergistic activation of the *EDNRB* promoter in combination with Sp1 (a 5.5-fold increase). We again confirmed the expression of each SOX10 mutant protein in HeLa cells (data not shown). These results also indicate the involvement of SOX10 and Sp1 in the activation of the *EDNRB* promoter.

We next examined the dominant-negative effect of each SOX10 mutant protein on the transactivation of *EDNRB* promoter achieved by a combination of SOX10 and Sp1 (Fig. 3C), because it has been reported that the SOX10 mutant protein, lacking the C-terminal domain, has a dominant-negative effect in a transient assay of the reporter containing three copies of the SOX10 consensus binding sites (*46*). Among the SOX10 mutant proteins examined, only Q377X did not show the dominant-negative effect, suggesting that Q377X may function as an activator of the *EDNRB* promoter. Taken together, these results suggest the remarkable differences in functional consequences of SOX10 mutations (Table 1).

DISCUSSION

The present study has demonstrated the impaired functions of SOX10 mutant proteins, associated with WS4 (E189X, Q377X, and 482ins6) or YDBS (S135T), as judged by the functional analyses of the *EDNRB* or *MITF-M* promoter. Importantly, the degree of the impaired function varies depending on the mutation or the target gene promoter (Table 1). Thus, the auditory-pigmentary abnormalities of the SOX10-associated WS4 and YDBS are caused at least in part by the reduced expression of EDNRB and/or MITF-M (Fig. 4).

Different Functional Consequences of Truncated SOX10 Proteins—We compared the functions of the two truncated SOX10 proteins, Q377X and E189X, each of which is associated with WS4. It is noteworthy that the functional consequences of Q377X and E189X proteins appear to be inconsistent with the severity of the phenotypes of the patients with each mutation (Table 1). The seemingly discordant results suggest the presence of complex regulatory network for expression of EDNRB.

Q377X protein has been reported as a dominant-negative form due to the lack of the C-terminal transactivation domain, and is associated with a severe phenotype of WS4, such as peripheral demyelinating neuropathy, and central dysmyelinating leukodystrophy in addition to long segment aganglionosis (*47, 48*). However, Q377X protein did not show the dominant-negative effect on the *EDNRB* promoter (Fig. 3C), suggesting that Q377X can function as an activator of the *EDNRB* promoter. It is therefore conceivable that the phenotypes caused by Q377X may be due to the reduced expression of other target genes for SOX10, such as c-RET (*49*).

On the other hand, the phenotype of the E189X mutation appears milder than that of the Q377X mutation (*46*) (Table 1), despite the severely impaired function of the E189X protein. In this context, it is noteworthy that E189X protein with a dominant-negative effect (see Fig. 3C) may be only marginally expressed *in vivo* due to the degradation of its mRNA by nonsense-mediated

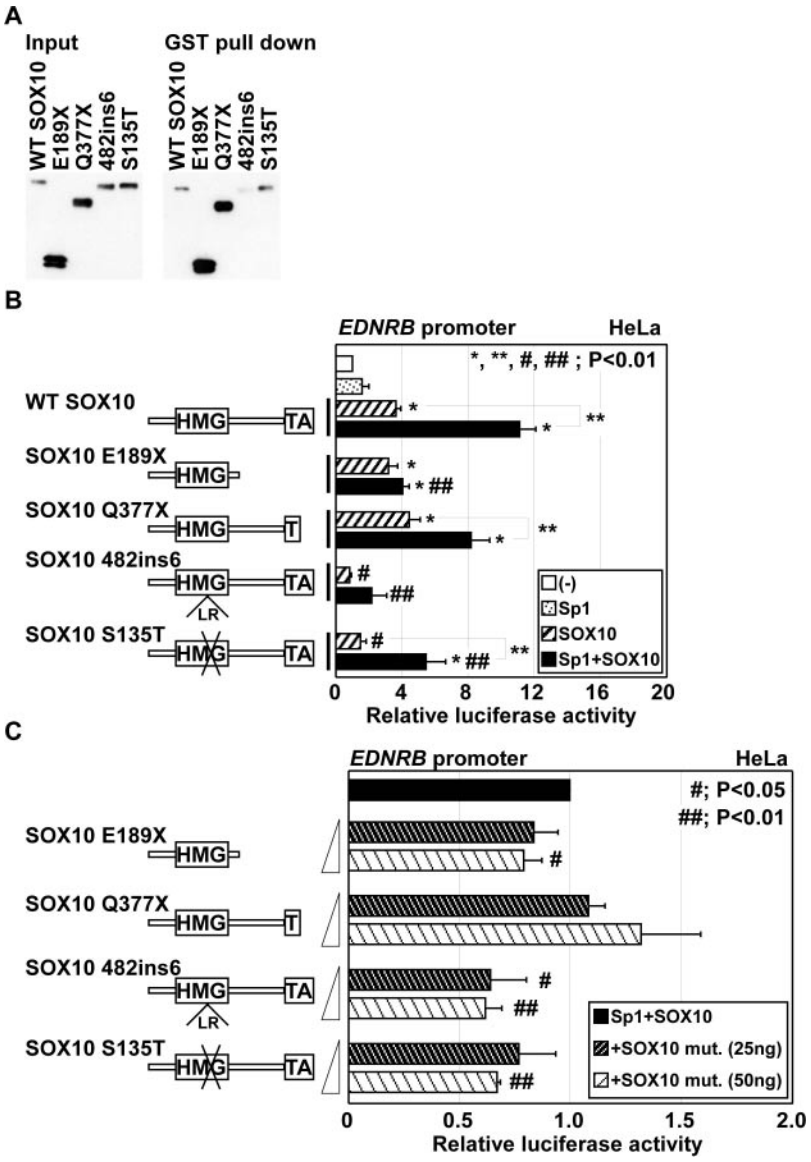

Fig. 3. **Functional differences of the SOX10 mutant proteins for the *EDNRB* promoter.** (A) Interaction of SOX10 with Sp1 *in vitro*. COS-7 cells were transiently transfected with each FLAG-tagged SOX10 expression vector, shown on the top of the lane (the left panel, the input proteins). The nuclear proteins that bound to GST-Sp1 were subjected to western blot analysis with an anti-FLAG antibody (the right panel). (B) Effects of SOX10 mutant proteins on the *EDNRB* promoter. Each FLAG-tagged SOX10 expression vector used was shown in the left. HeLa cells were co-transfected with pGL3-E (-3022) and empty vector (an open bar), Sp1 expression vector (a dotted bar), each FLAG-tagged SOX10 mutant expression vector (a shadow bar), or both of Sp1 and each FLAG-tagged SOX10 mutant expression vector (a filled bar). Other conditions are the same as indicated in Fig. 2. The activity with * is significantly higher than the value obtained with empty vector, $p < 0.01$. The activity with ** is significantly higher than the value obtained with each SOX10 expression vector, $p < 0.01$. The activity with # is significantly lower than the value obtained with wild-type SOX10 expression vector, $p < 0.01$. The activity with ## is significantly lower than the value obtained with co-transfection of Sp1 expression vector and wild-type SOX10 expression vector, $p < 0.01$. (C) Dominant-negative effect of SOX10 mutant proteins on the transactivation of *EDNRB* promoter achieved by SOX10 and Sp1. HeLa cells were co-transfected with pGL3-E (-3022), Sp1 expression vector (50 ng), SOX10 expression vector (50 ng), and each FLAG-tagged SOX10 mutant expression vector (25 or 50 ng) or the empty vector. Other conditions are the same as indicated in Fig. 3B. The activity with # or ## is significantly lower than the value obtained with both Sp1 and SOX10, $p < 0.05$ or $p < 0.01$, respectively.

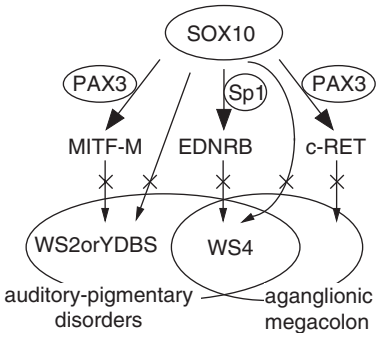

Fig. 4. **Proposed model for transcriptional network of the WS-related genes.** SOX10 regulates the expression of MITF-M, EDNRB, and c-RET by interacting with Sp1 or PAX3, and contributes to the development of melanocytes and enteric nervous system. Mutations at each gene (marked with a cross) cause abnormality of melanocytes or enteric nervous system, which is manifested as auditory-pigmentary disorders (Waardenburg syndrome, WS and Yemenite deaf-blind hypopigmentation syndrome, YDBS) or aganglionic megacolon, respectively.

decay, which is a surveillance system for removing nonfunctional mRNAs and degrades transcripts containing nonsense codons, which are followed by at least one intron (*46*). Thus, the E189X allele may mimic a loss-of-function mutant, thereby leading to a mild form of megacolon.

Functional analyses of E189X and Q377X proteins suggest that the region of SOX10 (189 to 377 amino acid residues), which is not present in E189X protein, is required for the synergistic transactivation of *EDNRB* promoter with Sp1 (Fig. 3). In fact, the region between 232 and 306 amino acid residues shows 61% similarity to the equivalent region of mouse Sox8 (215 to 293 amino acid residues), which is required for the transactivation activity (*50*). Thus, Q377X protein retains a second transactivation domain. The synergistic transactivation of *EDNRB* promoter by SOX10 and Sp1 may require a certain transcription factor and/or co-factor, which interacts with the second transactivation domain of SOX10.

Different Functional Consequences of Mutations at the HMG Box of SOX10—Sp1 may partially rescue the

impaired function of SOX10 S135T mutant protein in the activation of the *EDNRB* promoter, which may contribute to the phenotypic variations between WS4 and YDBS. Notably, S135T protein, lacking the DNA-binding activity, activates the *EDNRB* promoter, by interacting with Sp1 (Fig. 3), indicating that the impaired function of SOX10 could be compensated for in part by Sp1.

It has also been reported that S135T protein, but not 482ins6 protein, interacts with Pax3 and activates the c-*RET* promoter (*44, 51*). The mutation in the c-*RET* gene is responsible for the pathogenesis of aganglionic megacolon (*49*). Moreover, 482ins6 protein failed to show the collaboration with PAX3 on the *MITF* promoter (Fig. 2D). Thus, S135T protein activates both the *EDNRB* and *c-RET* promoters by interacting with Sp1 or PAX3, which accounts for the absence of megacolon in YDBS (Fig. 4). As summarized in Table 1, the expression deficiency of c-*RET* in E189X, Q377X and 482ins6 seems to be the main reason of megacolon phenotype (*44, 51*).

On the other hand, melanocyte abnormalities seen in YDBS associated with S135T protein are due to not only reduced transactivation of *EDNRB* gene but also that of *MITF* gene (Figs. 2C and 3B). The transactivation ability of S135T protein on the *MITF-M* promoter is lower than that of wild type SOX10, but higher than 482ins6 protein (Fig. 2C). It has also been reported that either 482ins6 or S135T protein was unable to activate the *MITF-M* promoter synergistically in combination with PAX3 (*44*). Thus, melanocyte abnormalities associated with the mutation of SOX10 may be in part due to the reduced expression of MITF-M, because haploinsufficiency of MITF is a mechanism that causes WS (*1*). In this context, MITF-M may function as a self-regulator of transcription from the *MITF-M* promoter, thereby maintaining a threshold level of MITF-M during a certain sensitive stage of development (*52*).

Regulatory Network for Transcription of the EDNRB Gene—The WS4 patient with 482ins6 mutation is deaf and has short segment aganglionosis (*3*). The YDBS patients with S135T protein also show the deafness but do not manifest aganglionosis and ocular defects except for nystagmus (*18*). The transactivation ability of S135T protein on the *EDNRB* promoter may be sufficient for the development of enteric nervous systems, but the transactivation ability on *MITF-M* promoter may not be sufficient for the development of the melanocytes at least in the skin and inner ear. In the mouse model of a recessive form of WS4, associated with the *Ednrb* gene mutation (*53*), the stria vascularis in the cochlea lacks intermediate cells (melanocytes). In this context, we have shown that both Sox10 and Mitf mRNAs are expressed in the migrating melanoblasts, precursors to intermediate cells, of mouse embryo (*54*). In addition, SOX10 is important in the correct arrangement of sensory cells in the organ of Corti (*54, 55*). These notions suggest that the development of the cochlea is ensured by the collaboration of SOX10, MITF, and EDNRB.

The interaction of SOX10 with Sp1 could account for the phenotypic difference between WS4 and YDBS, as learned from the property of S135T protein. We therefore suggest that Sp1 may represent a modifier for WS4 (*3, 4*). In one family case of WS4, the proband and his sister are heterozygous for the Q377X mutation, but only the proband has an aganglionic megacolon (*4*). These observations suggest the presence of modifier genes for the *EDNRB* gene, and the expression of the modifier genes may be influenced by the environmental factors, thereby leading to the phenotypic variability of WS4. One of such modifiers might be Sp1, the function of which is modulated by several growth factors (*56*) or metals (*57*).

In summary, we have shown the different effects of each SOX10 mutant protein on the *EDNRB* or *MITF-M* promoter, which could account for the phenotypic differences between WS4 and YDBS (Fig. 4). Furthermore, we suggest that Sp1 might be one of the modifiers of WS4. The functional analysis of the mutant proteins of WS-related genes will help us understand the relationship between the genetic disorders and the phenotypes.

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