ABSTRACT

The transcription factor Sox5 has previously been shown in chicken to be expressed in early neural crest cells and neural crest-derived peripheral glia. Here, we show in mouse that Sox5 expression also continues after neural crest specification in the melanocyte lineage. Despite its continued expression, Sox5 has little impact on melanocyte development on its own as generation of melanoblasts and melanocytes is unaltered in Sox5-deficient mice. Loss of Sox5, however, partially rescued the strongly reduced melanoblast generation and marker gene expression in Sox10 heterozygous mice arguing that Sox5 functions in the melanocyte lineage by modulating Sox10 activity. This modulatory activity involved Sox5 binding and recruitment of CtBP2 and HDAC1 to the regulatory regions of melanocytic Sox10 target genes and direct inhibition of Sox10-dependent promoter activation. Both binding site competition and recruitment of corepressors thus help Sox5 to modulate the activity of Sox10 in the melanocyte lineage.

INTRODUCTION

Many transcription factors of the Sox protein family are widely expressed in the vertebrate embryo and represent important developmental regulators (1,2). Several subgroups can be distinguished, one of them is the SoxD group. SoxD proteins do not only share a high degree of amino acid similarity, but also a number of biochemical features that make them unique among Sox proteins. For one, SoxD proteins exist in several isoforms that are generated by alternative splicing from rather large genes with multiple exons (3). This is uncommon for Sox proteins which are usually encoded by intronless genes or compact genes with few exons (2). As a consequence, SoxD proteins exist in short and long isoforms. Only the long isoforms contain a characteristic coiled-coil domain that allows these proteins to homodimerize or heterodimerize with other SoxD proteins (3). In fact, the long SoxD isoforms are the only Sox proteins known to exist as dimers in solution. SoxD proteins furthermore differ from other Sox proteins in that they do not appear to have a typical transactivation domain (3) arguing that SoxD proteins influence transcription in other ways.

Vertebrates possess the three highly related proteins Sox5, Sox6 and Sox13 (3–5). Each of them is expressed widely during embryogenesis. All three SoxD genes have been deleted in the mouse and found to influence a wide range of developmental processes including the development of chondrocytes, oligodendrocytes, corticofugal neurons, erythrocytes, T lymphocytes, the notochord and the heart (6–13). During some of these events, SoxD proteins primarily function as modulators of the SoxE proteins Sox9 and Sox10 (3,7,9), whereas this is not the case in other developmental processes (10,13). Sox5, in particular, has been found to influence chondrocyte development by cooperating with and reinforcing the activity of Sox9 on several of its chondrocytic target genes (3,14), whereas its impact on oligodendrocyte maturation appears to be primarily due to its ability to counteract the activity of Sox10 on several of its oligodendroglial target genes (9). In agreement with its numerous functions, Sox5 cannot be deleted in mice without severe consequences and Sox5-deficient mice die at birth because of respiratory distress (7).

In the chicken, Sox5 is also expressed in the neural crest (15). In the cranial neural crest, Sox5 was found in premigratory and migratory neural crest cells, but also in some neural crest derivatives, in particular in the glial components of the peripheral nerves and ganglia (15,16). Its importance for neural crest development had furthermore been inferred from ectopic expression in the electroporated neural tube, where it promoted the generation of additional neural crest cells. Sox5 also occurs in neural crest cells of the mouse (C.S. and M.W., unpublished data). Whether its role in the neural crest involves a functional interplay with Sox9 or Sox10 has not been analyzed so far. It has to be noted, however, that both Sox9 and Sox10 are strongly expressed in the neural crest and regulate various phases of neural crest development. Sox9, for instance, is involved in defining the premigratory neural crest cell, ensures its survival and allows its epithelial-to-mesenchymal transition, whereas Sox10 is
essential for survival of migratory neural crest cells and maintenance of their pluripotency (17,18). Additionally, both SoxE proteins influence several cell fate decisions in the neural crest at later stages of development, with Sox9 being important for acquisition of the chondrocytic fate of cranial neural crest cells and Sox10 driving glial and melanocytic specification (19,20).

Melanocytes are derived from neural crest cells that migrate on a dorsolateral pathway immediately below the epidermis. Cells specified to become melanoblasts are recognizable by their expression of several markers, including the receptor tyrosine kinase c-Kit, the dopaminechrome tautomerase Dct and the basic helix-loop-helix transcription factor Mitf (21). In fact, Mitf is the master regulator of melanocyte development and the onset of its expression during development distinguishes a migrating neural crest cell from a melanoblast (21). Induction of Mitf expression in the specified melanoblast is dependent on Sox10 and Pax3, thus explaining why Sox10 is essential for melanocyte development (19,22–24). There is good evidence that Mitf is a direct target gene of Sox10 and probably also of Pax3 in neural crest cells (25–29). Once induced, Mitf has to be stimulated in its activity by ERK-dependent phosphorylation upon c-Kit activation (30). One of the earliest genes induced by Mitf in melanoblasts is Dct and this induction occurs in synergy with Sox10 (31–34). In contrast to the functions of Sox10, Mitf and c-Kit in melanoblasts, those of Det are not known, but seem to be nonessential and different from its later role in melanin production in differentiated melanocytes (35).

Here, we report that Sox5 is expressed in melanoblasts during mouse development and analyzes its function, in particular, in relation to Sox10 as a known major regulator of melanocyte development.

**MATERIALS AND METHODS**

**Animal husbandry, genotyping, tissue preparation, immunohistochemistry and in situ hybridization**

Starting with Sox10+/−/lacZ mice (19) on a pure C3HeB/FeJ genetic background and Sox5+− mice (7) backcrossed for at least four generation on the same C3HeB/FeJ background, double heterozygous mice were obtained whose intercrossing or breeding with Sox5+− mice generated offspring of the expected genotypes determined 48 h posttransfection (41). In select plasmids, regions corresponding to positions 966–984 or 1135–1153 of mouse Sox10 (Genbank accession number BC023356), to positions 1415–1433 of mouse Sox13 (Genbank accession number NM_011444.1) and to positions 1486 to +97 of the Dct promoter (positions −685 to +443; 32) were used. Effector plasmids corresponded to pCMV5-based expression plasmids for Sox10, the long and short isoforms of Sox5 (9,39,40), anti-Sox13 guinea pig antiserum (1:1,000 dilution; generated against amino acids 566–588 of mouse Sox13 according to accession number Q04891), anti-Sox6 guinea pig antiserum (1:1000 dilution; 9), anti-Sox5 guinea pig antiserum (1:500 dilution; 9), anti-Sox5 rabbit antiserum (1:4000 dilution; 15), anti-L-Sox5 rabbit antiserum (1:500 dilution, gift of V. Lefebvre, Lerner Institute, Cleveland, Ohio), anti-Sox13 rabbit antiserum (1:2000 dilution; Chemicon, Hofheim, Germany) and anti-Mitf rabbit antiserum (1:2000 dilution, gift of H. Arnheiter, NIH, Bethesda). Secondary antibodies conjugated to Cy2, Cy3 (Dianova, Hamburg, Germany) or Alexa (Molecular Probes, Göttingen, Germany) immunofluorescent dyes were used for detection. Immunofluorescence was detected and documented with a Leica inverted microscope (DMIRB) equipped with a cooled SPOT CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA).

For whole-mount in situ hybridizations, embryos were fixed overnight at 4 °C in 4% paraformaldehyde, dehydrated, bleached and rehydrated. In situ hybridization was performed essentially as described with DIG-labeled antisense riboborbes for c-Kit, Dct and Mitf (19,25). All steps except probe hybridization and final colorimetric detection were performed automatically on a Biolane HTI (Hölle & Hütter AG, Tübingen, Germany). In situ hybridizations were analyzed and documented with a Leica MZFLIII stereomicroscope equipped with an Axiocam (Zeiss, Oberkochem, Germany).

**Cell culture, RT–PCR and luciferase assays**

Neuro2a neuroblastoma cells were maintained in DMEM containing 5% FCS, and B16 melanoma cells were grown in DMEM containing 10% FCS. Both cell lines were transfected using Superfect reagent (Qiagen, Hilden, Germany).

For luciferase assays, cells were transfected transiently in duplicates in 24-well plates or 3.5-cm dishes with 500 ng of luciferase reporter plasmid and 100 ng of effector or shRNA expression plasmids per well/dish if not stated otherwise. Luciferase reporters containing the Mitf promoter (positions −1486 to +97; 25) or the Dct promoter (positions −685 to +443; 32) were used. Effector plasmids corresponded to pCMV5-based expression plasmids for Sox10, the long and short isoforms of Sox5 (9,39,40), myc-tagged HDAC1 (gift of S. Minucci, European Institute of Oncology, Milan, Italy) and HA-tagged CtBP2 (gift of G. Chinnadurai, St Louis University School of Medicine, St Louis, USA). For shRNA expression plasmids, regions corresponding to positions 966–984 of mouse Sox10 (Genbank accession number BC023356), to positions 1415–1433 of mouse Sox5 (Genbank accession number NM_011444.1) and to positions 1135–1153 of mouse Sox6 (Genbank accession number U32614) were each inserted between the BglIII and XhoI sites of the pSUPER.neo + gfp vector (Oligoengine, Seattle, USA). Scrambled versions were inserted into pSUPER.neo + gfp as controls.

For luciferase assays, cells were harvested and activities determined 48 h posttransfection (41). In select
experiments, 50 ng pCMV5-lacZ was additionally co-transfected to normalize luciferase activities to β-galactosidase activities. This normalization did not alter the obtained results.

Immunocytochemistry was performed on B16 melanoma cells at 30% confluency before transfection or 24 h after transfection with shRNA expression plasmids using the anti-L-Sox5, anti-Sox6, anti-Sox9, anti-Sox10, anti-Sox13, anti-Mitf and secondary antibodies described above at 5-fold higher dilution. Transfected cells were identified by GFP autofluorescence.

RNA was prepared from B16 melanoma cells and spinal cords of 7-day old mice using Trizol reagent (Invitrogen, Karlsruhe, Germany). After reverse transcription to cDNA, semiquantitative PCR was performed to detect products specific for Sox5 (5'-AGCCCCACATAAAG CGTCCAAAT-3' and 5'-GGTCCCTCCTCCTCATC GTA-3'), Sox6 (5'-AGCAGAGCCTGTGAAGGATTTC TCT-3', and 5'-CTTTGCTTCTCCTTG CGTCC-3'), Sox9 (5'-GGACAGACTCACTTCCCTCC-3' and 5'-TGCTGTCCTT GCATCCACAC-3'), Sox10 (5'-GTGAGATGGGAA CCCAGAGCAC-3', and 5'-CCCCGAGCCAGCT CGGAG-3'), Sox13 (5'-AGCAGATCTTGGTTC TCG-3' and 5'-GGAGACTGCAGGTATTGATG-3'), Dct (5'-TTCCAGAACTCAGGAGTGG-3' and 5'-CAT TGCTCACGGTCATTAATACTACTGGAACTA-3'), Mitf (5'-AAGGAGGAGC GAAAAAGGACT-3' and 5'-GTGTTGCTGTAAGGTTG ATGG-3') or β-actin (5'-CCTGGGCATGGAGTCCTG-3' and 5'-GGAGCAGAATGGATCTGAT CCTC-3').

Extract preparation, electrophoretic mobility shift assay and western blot
Extracts from untransfected B16 cells (10-cm dishes) or Neuro2a cells transfected with expression plasmids for the long and short Sox5 isoforms, Sox10, or the carboxy-terminally shortened MIC version of Sox10 (amino acids 1–189) were prepared as described (42). With these extracts, electrophoretic mobility shift assay (EMSA) was performed in the presence of poly(dGdC) as unspecific competitor using 32P-labeled oligonucleotides containing the dimeric binding site 5′/C0 (5′-GGGGAGGCTCT ATAAAAGGCCCTTTGACGGAATGC-3', positions −310 to −282) and the monomeric binding site S1 (5′-GGGGTCTAGTGCATAACAAAGGAGATTCTCTC-3', positions −150 to −121) from the Dct promoter, or the Sox10 binding site 5′ from the Mitf promoter (5′-GGGAGGACATTTGGACATTTG-3', positions −270 to −242) as probes. For supershift experiments, anti-Sox10 or anti-Sox5 antisera were added.

Extracts were also subjected to SDS–PAGE. Western blotting was performed using specific antisera against Sox5 (1:3000 dilution), Sox10 (1:5000 dilution) and acetylated α-tubulin (1:2000 dilution; Sigma, Taufkirchen, Germany) as primary antibodies, and horse-radish peroxidase-coupled secondary antibodies with enhanced chemiluminescence.

Chromatin immunoprecipitation
Chromatin immunoprecipitation assays on B16 cells and transfected Neuro2a cells were performed as described (43). Briefly, cellular protein and genomic DNA were cross-linked by treatment with 1% formaldehyde before chromatin extraction and sonication to an average fragment length of 300–600 bp. Immunoprecipitations were performed overnight at 4°C using polyclonal IgG against Sox10 and Sox5 from guinea pig, as well as mouse monoclonals against HDAC1 (clone 2E10, Millipore, Schwalbach, Germany), CtBP2 (clone 16, BD Transduction Laboratories, Heidelberg, Germany) or the HA epitope (clone 12CA5). DNA was purified from precipitates after cross-link reversal and subjected to PCR. For detection of the Mitf promoter (positions −369 to −125), 5′-TTGGCCATT AGGGAAATGCTC-3′ and 5′-CAGTTTCCCTGCTGAGC TTGA-3′ were used as primers in 3 cycles of standard PCR using an annealing temperature of 60°C. A control region from the Mitf upstream region (positions −5993 to −5641) was probed with 5′-TTGGCTCTTATGCTGT TCTCT-3′ and 5′-GCTCGGAAGGCTACATATC-3′ as primers. The 5′-CAGGAGGATGCTAAATTGTG-3′ and 5′-GACTTCCCCAGTGGCATAAT-3′ were employed to detect the Dct promoter (positions −457 to −271), primers 5′-GGACACACCACATCCTGTAT-3′ and 5′-ACGATTGGTCTGAGGAAGT-3′ to amplify a distal fragment from the Dct upstream region (positions −4080 to −39738).

RESULTS
Sox5 is expressed in the melanocyte lineage of the mouse embryo
Sox5 had previously been detected in the early migrating neural crest of chicken (15) and is similarly expressed in the mouse neural crest (C.S. and M.W., unpublished data). To study whether Sox5 expression continues in those migrating neural crest cells that become specified to melanoblasts, we performed colabeling studies on mouse embryos at 11.5 dpc (Figure 1). Using antibodies directed against Sox10, migrating neural crest-derived cells were identified in the trunc region immediately below the epidermis (Figure 1B, C, F and G). These cells were also labeled by Sox5-specific antibodies including those that recognized all Sox5 isoforms (Figure 1A and C) and those that specifically recognized the long Sox5 isoforms (Figure 1E and G). To confirm that these Sox5-positive neural crest-derived cells corresponded to melanoblasts, we performed additional colabeling with antibodies directed against Mitf (Figure 1J). Significant amounts of Sox5 were present in Mitf-positive cells (Figure 1J) indicating that Sox5 is indeed expressed in the melanocyte lineage (Figure 1K and L). The melanocyte lineage thus represents one of several cell types in which Sox5 and Sox10 are coexpressed (9,16).

Whereas most Mitf-positive cells contained Sox5 at 10.5, 11.5 and 12.5 dpc (Figures 1I–L and 2A–H), Sox5 was absent from most Mitf-expressing cells at 16.5 dpc and postnatal day 3 (Figure 2I–P). This argues that Sox5 is expressed during embryonic development in melanoblasts, but disappears from these cells with their differentiation to melanocytes.
As Sox5 has previously been found in chondrocytes and oligodendroglial cells to be coexpressed with the closely related Sox6 (9,44,45), we also assessed Sox6 expression. Between 10.5 and 12.5 dpc, Sox6 amounts in melanoblasts were as low as in many cells of the surrounding tissue, and significantly less than those in epidermal cells (Figure 1M and data not shown). With this low expression levels, melanoblasts are clearly not a prime site of Sox6 expression (Figure 1M–P). Sox13, as the third SoxD protein was not detected in melanoblasts (Figure 1Q–T). Therefore, we considered it unlikely that Sox6 and Sox13 play major roles in melanoblasts, and concentrated on Sox5.

Sox5 deletion has no major impact on melanoblast development by itself but modulates the consequences of Sox10 heterozygosity

Taking the widespread expression in melanoblasts into account, we next asked whether loss of Sox5 would influence the early stages of melanocytic development. Whole-mount in situ hybridizations were performed on Sox5+/− embryos at 10.5 and 11.5 dpc to follow melanoblast development and results were compared with age-matched wild-types (Figure 2 and data not shown). Care was taken that analyzed embryos throughout our studies were in a very similar stage of development and had a comparable genetic background. Using c-Kit, Dct and Mitf as three independent markers of the early melanocyte lineage, we failed to detect any significant difference in the appearance or migration pattern of melanoblasts in Sox5+/− mice (compare Figure 3A, E, I and M to Figure 3B, F, J and N). Melanoblast numbers were also comparable between Sox5+/− embryos and their wild-type littermates (Figure 3Q and R). The fact that the absolute number of melanoblasts was twice as high with Dct as with Mitf as probe, simply reflects the different sensitivities of the respective probes. Distribution and number of melanocytes were also normal in Sox5+/− mice at the time of birth (data not shown). Our analyses thus revealed that Sox5 is dispensable for specification of melanoblasts and the consecutive phases of melanocyte development.

This contrasts dramatically with the previously reported impact of Sox10 on development of the melanocyte lineage (19,22,46). Already loss of a single Sox10 allele led to a severe impairment of early development of the melanocyte lineage that only partially recovered at later times. In accord with previous findings, all melanocyte markers were dramatically reduced in Sox10+/−/lacZ embryos at
10.5 dpc (Figure 3C, G, K and O). The fact that Dct expression was affected even stronger than that of the other two markers (compare Figure 3Q to Figure 3R), had been previously observed and was attributed to direct dependence of Dct expression on high Sox10 levels in the mouse (19,22,31–33).

We used the Sox10+/lacZ embryos to ask whether a role of Sox5 in melanocyte development could be visualized on this sensitized background. Therefore we generated Sox5+/−, Sox10+/lacZ double mutant embryos and compared development of the melanocyte lineage in this genotype with that in the two single mutants.
Interestingly, Sox5 did not aggravate, but rather alleviated the melanocytic phenotype of the Sox10+/lacZ embryos, although melanoblast numbers remained significantly below those in the wild-type and Sox5-deficient embryos (Figure 3D, H and P). Quantification revealed that the number of Dct-positive cells recovered from 2% to 8% of wild-type levels (Figure 3Q). For Mitf, recovery was from 30% to 70% of wild-type levels (Figure 3R). A similar increase of melanoblast numbers in the Sox5−/−, Sox10+/lacZ double mutant relative to Sox10+/lacZ embryos was also detected at 11.5 dpc (data not shown).

The most parsimonious explanation for this finding is that Sox5 normally counteracts the activity of Sox10 during early development of the melanocyte lineage so that its loss leads to a partial phenotypic rescue in Sox10+/lacZ embryos. Rates of proliferation and apoptosis were furthermore comparable for Mitf-expressing cells in all genotypes (data not shown) arguing that Sox5, like Sox10, already has an influence on melanocyte specification.

As observed previously, melanoblasts were completely absent in Sox10−/−/lacZ embryos (19). Additional loss of Sox5 did not lead to a recovery of melanoblasts in Sox5−/−, Sox10+/lacZ embryos confirming that some amount of Sox10 must be present for Sox5 to exert its function (data not shown).

**Sox5 has the capacity to bind to Sox10 response elements in melanocytic target genes of Sox10**

Sox10 has previously been shown to activate Mitf and Dct as its target genes (25–29,32–34). Depending on the studied organism, Mitf gene activation has been proposed to be one of the essential functions or the sole essential task of Sox10 during melanocytic development (23,31). Multiple binding sites for Sox10 have been mapped within the Mitf and Dct promoters. These sites have been found to differentially contribute to overall promoter activation. In the Dct promoter, the S1 and the S4/4′ sites have been reported to be mainly responsible for the Sox10-dependent induction (32). The same holds true for site 5 in case of the Mitf promoter (25). The S1 site from the Dct promoter and the site 5 from the Mitf promoter are furthermore representative of response elements recognized by Sox10 monomers, whereas S4/4′ allows binding of Sox10 dimers as evident from the mobility of the Sox10-containing complexes on these sites in EMSA (Figure 4A) (25,32).

We asked whether Sox5 has the capacity to recognize these response elements. When EMSA were performed with the long isoform of Sox5 that we had detected in melanoblasts at 10.5 dpc, binding was detected to all three sites (Figure 4A). From the comparable mobility of the Sox5-containing complex on site 5 and S1 relative to the S4/4′ site, it can furthermore be concluded that the L-Sox5 isoform binds to all sites as a dimer in agreement with its constitutive dimerization in solution (3).

As both Sox5 and Sox10 bound to the same response elements in the Mitf and Dct promoters, we analyzed whether simultaneous binding to these sites can occur or whether binding is mutually exclusive. For this purpose, Sox5 and Sox10 were together incubated with each of the three sites (Figure 4B–D). For better visualization, a carboxyterminally truncated Sox10 version was used. In EMSA, only complexes were observed whose mobility corresponded to the Sox5- or the Sox10-containing complex. Additional supershift experiments with Sox5- and Sox10-specific antibodies confirmed that the complexes with Sox5- or Sox10-specific mobility indeed contained only one of the two Sox proteins (Figure 4B–D). Similar results were obtained when full-length Sox10 was used instead of the truncated version (data not shown).

From the unchanged composition of the complexes and our failure to obtain novel complexes with intermediate or higher mobility, a mutually exclusive mode of binding is concluded. This was also confirmed in titration experiments in which increasing amounts of Sox10 were capable of displacing a fixed amount of Sox5 from S4/4′ (Figure 4E) just as increasing amounts of Sox5 were capable of displacing a fixed amount of Sox10 without the appearance of any additional complex (Figure 4F).

**Sox5 prevents Sox10 from inducing the promoters of its melanocytic target genes**

We next addressed the ability of both Sox proteins to influence the activities of the Mitf and Dct promoters in luciferase reporter assays. Whereas Sox10 robustly activated both promoters in transiently transfected Neuro2a cells, no such activation was observed for the long Sox5 isoform (L-Sox5) over a broad range of concentrations (Figure 5A and B and data not shown). This agrees with previous findings that all Sox5 isoforms lack a classical transactivation domain (3). When reporter genes were cotransfected simultaneously with both Sox proteins, Sox10 furthermore lost its ability to efficiently activate either the Dct promoter (Figure 5A) or the Mitf promoter (Figure 5B). We thus conclude that the long Sox5 isoform counteracts the transcriptional activity of Sox10 on its melanocytic target gene promoters in heterologous cell lines. Qualitatively similar results were also obtained in transiently transfected B16 melanoma cells (Figure 5C and D), although Sox10-dependent activation rates were lower, likely because of the presence of endogenous Sox10 in these cells (47) (see also Figure10A and B).

Because Neuro2a cells have a neural crest origin and at the same time provide a Sox10-free background, we continued our luciferase reporter assays in these cells. When constant amounts of Sox10 were challenged with increasing amounts of Sox5, Sox10-dependent activation of both Dct and Mitf promoters was counteracted by the long Sox5 isoform in a concentration-dependent manner (Figure 5E and F). Similar to the long isoform, the short Sox5 isoform did not activate Dct promoter or Mitf promoter on its own (Figure 5G and H). Interestingly, however, the short Sox5 isoform failed to inhibit Sox10-dependent activation. We thus conclude that the ability to modulate Sox10 activity on these promoters is restricted to the long isoform of Sox5 which is present in melanoblasts.
Sox5 is coexpressed with Sox10 in B16 melanoma cells

Using the B16 cell line, we next investigated whether Sox5 is also expressed in melanoma cells. First, we searched for the presence of Sox5 transcripts. RT–PCR studies revealed that B16 cells not only contained Sox10, Dct and Mitf transcripts, but also significant levels of Sox5 transcripts (Figure 6A). Sox6 and Sox9 transcripts were also detected (Figure 6A) in agreement with previous studies (48,49). Sox6 expression is thus clearly higher in B16 melanoma than in melanoblasts at 11.5 dpc. The same discrepant expression was also observed for Sox9 which is strongly expressed in B16 melanoma, but virtually absent from melanoblasts in mouse embryos (Figure 1U–X), again in good agreement with previously published data (49). The presence of Sox6 and Sox9 in B16 melanoma constitutes a significant complication as functional

Figure 4. Sox5 recognizes binding sites for Sox10 in the Dct and Mitf promoters. (A) Binding of the long Sox5 isoform to bona fide Sox10 recognition elements was studied in EMSA with the monomeric Sox10 binding site S1 and the dimeric site S4/4' from the Dct promoter and the binding site 5 from the Mitf promoter as probes (25,32). Extracts from transfected Neuro2a cells served as source for full-length Sox10 and Sox5. Unspecific complexes (ns) were identified as those that also appeared with extract from mock-transfected Neuro2a cells (C). Each of the specific complexes is labeled on the right side of the panel for the presence of Sox5, Sox10 dimers (Sox10 D) or Sox10 monomers (Sox10 M) in the respective complexes. The ‘−’ depicts no extract added. Using the S1 (B), S4/4' (C) and Mitf (D) probes, EMSA was performed with extracts from transfected Neuro2a cells containing the carboxyterminally truncated MIC variant of Sox10 and/or Sox5. Addition of antibodies directed against Sox10 (α-Sox10) or Sox5 (α-Sox5) during the incubation period was used to identify the complexes containing either Sox protein. Supershifted complexes are marked by asterisks on the right side of the panels. No heteromeric complexes were observed. For titration experiments, a fixed amount of Sox5 and S/S4' were challenged with increasing amounts of Sox10 (E), or a fixed amount of Sox10 and S/S4' were reciprocally incubated with increasing amounts of Sox5 (F). As higher amounts of extract have to be used to obtain a Sox5-specific complex than a Sox10-specific complex, unspecific complexes become apparent with increasing Sox5 amounts.
compensation between closely related Sox proteins is possible in this cell line. In contrast, such a functional compensation is unlikely \textit{in vivo}, as melanoblasts only express Sox5 and Sox10 in significant amounts. Sox13 was similarly absent from 11.5 dpc melanoblasts and B16 melanoma cells (Figure 1Q–T and Figure 6A). Sox5 was also detectable on the protein level in B16 melanoma cells by western blotting (Figure 6B). As judged by its molecular weight, most of the Sox5 protein corresponded to the L-Sox5 isoform (Figure 6B). This Sox5 protein was also responsible for one of the complexes obtained in EMSA with the Mitf site 5 and B16 extract as judged by antibody supershift experiments (Figure 6C). The Sox5-specific complex was significantly less intense than the Sox10-containing complex, which furthermore exhibited a higher mobility as predicted from the previous results with ectopically expressed Sox proteins (see Figure 4A). This lower amount of the Sox5-specific complex could either be due to a lower affinity of Sox5 for the Mitf site in the context of the B16 extract or to lower amounts of the protein. Taking the results from western blotting into account (Figure 6B), lower amounts appear to be at least a contributing factor.

To study the occurrence of Sox proteins in B16 cells on a cellular level, we also performed co-immunocytochemistry. The vast majority of analyzed B16 cells indeed expressed Sox5 (compare Figure 7A and E to Figure 7D and H) as well as Sox6 (compare Figure 7I to Figure 7L) and Sox9 (compare Figure 7Q to Figure 7T), but not Sox13 (compare Figure 7M to Figure 7O and P). Importantly, B16 cells were also quite homogenous regarding their expression of Sox10 (Figure 7F, J, N and R). As a consequence, Sox5, Sox6 and Sox9 were all coexpressed in most B16 cells with Sox10 as well as Mitf (Figure 7C, G, K and S).

**Sox5 represses Sox10-dependent target gene expression in B16 melanoma cells**

Despite this complex Sox protein expression pattern, the impact of Sox5 on the expression of Sox10 target genes was assessed in B16 melanoma cells by transfection with shRNA vectors. Using GFP expressed from the IRES-EGFP cassette of the shRNA plasmid, we identified the transfected cells and analyzed the amount of Sox proteins in these cells relative to untransfected neighboring cells. These studies revealed that a Sox10-specific shRNA reduced the amount of Sox10, but not of Sox5 in B16 melanoma cells (Figure 8A, B, E and F), whereas a Sox5-specific shRNA likewise had no influence on the Sox6 amounts in transfected cells (Figure 9A and B). Furthermore, scrambled versions of the Sox5-specific or the Sox10-specific shRNAs did not change the level of either Sox protein and served as controls (Figure 8C, D, G, H, O, P, S and T). Concomitant with a reduction of endogenous Sox10 levels, there was also a dramatic reduction of Mitf amounts in >90% of all B16 cells transfected with a Sox10-specific shRNA (Figure 8I and J), thus
confirming Mitf as a Sox10 target gene (25–29,33). In contrast, >90% of B16 cells transfected with a Sox5-specific shRNA or the scrambled shRNA versions exhibited unaltered Mitf levels (Figure 8K, L, U, V, W and X). These experiments confirm on a cellular level that on its own endogenous Sox10, but not Sox5 influences Mitf expression in melanocyte-derived cells.

When B16 melanoma cells were simultaneously transfected with equal amounts of Sox5- and Sox10-specific shRNA vectors, we detected substantial amounts of Mitf in approximately two-thirds of the transfected cells (Figure 8Y and Z). We take this as evidence that a concomitant reduction of endogenous Sox5 levels relieves the impact of decreased Sox10 amounts on Mitf expression. The fact that Mitf levels differ substantially among transfected cells is probably caused by cell-to-cell variations in the residual amounts of Sox5 and Sox10. If, for instance, Sox10 levels are lowered by shRNA below threshold levels, concomitant Sox5 reductions probably remain without effect. Nevertheless, our results are compatible with a model in which Sox5 antagonizes Sox10 target gene activation in cells of melanocytic origin.

Analogous experiments were also carried out with a Sox6-specific shRNA vector. As expected, Sox6 levels were specifically reduced by the shRNA below threshold levels, concomitant Sox5 reductions probably remain without effect. Nevertheless, our results are compatible with a model in which Sox5 antagonizes Sox10 target gene activation in cells of melanocytic origin.

In contrast, >90% of B16 cells transfected with a Sox5-specific shRNA or the scrambled shRNA versions exhibited unaltered Mitf levels (Figure 8K, L, U, V, W and X). These experiments confirm on a cellular level that on its own endogenous Sox10, but not Sox5 influences Mitf expression in melanocyte-derived cells.

When B16 melanoma cells were simultaneously transfected with equal amounts of Sox5- and Sox10-specific shRNA vectors, we detected substantial amounts of Mitf in approximately two-thirds of the transfected cells (Figure 8Y and Z). We take this as evidence that a concomitant reduction of endogenous Sox5 levels relieves the impact of decreased Sox10 amounts on Mitf expression. The fact that Mitf levels differ substantially among

Figure 6. Sox5 is coexpressed with other Sox genes in B16 melanoma cells. (A) PCR experiments were performed on DNaseI-treated, reverse transcribed RNAs from B16 melanoma cells (B16) and spinal cords of 7-day old mice (P7), the latter serving as a positive control as most Sox genes are known to be expressed in this tissue. Transcripts were detected with mouse-specific primers for Sox5, Sox6, Sox13, Sox9, Sox10, Dct, Mitf and β-actin. Template-free control reactions were performed in parallel (--). (B) Equal amounts of extracts from B16 melanoma cells were subjected to SDS–PAGE and western blotting using antibodies directed against Sox5, Sox10 and acetylated α-tubulin. On the right side, the size of coelectrophoresed molecular weight markers are given. (C) EMSA was performed with the binding site from the Mitf promoter as probe and extracts from B16 melanoma cells. Addition of antibodies directed against Sox10 (α-Sox10) or Sox5 (α-Sox5) to the reactions during the incubation period was used to identify the Sox5- and Sox10-containing complexes, respectively. The ‘−’ depicts no extract added.

Figure 7. Sox5 is coexpressed on a cellular level with other Sox proteins and Mitf in B16 melanoma cells. Immunocytochemistry was carried out on B16 melanoma cells using antibodies against the long Sox5 isoform (A, C, F and G), Sox6 (I and K), Sox13 (M and O) and Sox9 (Q and S) (in red). B16 melanoma cells were additionally counterstained with antibodies directed against Sox10 (B and C) and Mitf (E, G, J, K, N, O, R and S) (all in green). Panels (A, B, E, F, I, J, M, N, Q and R) show single color recordings, panels (C, G, K, O, S) merged pictures. Nuclei of B16 melanoma cells were counterstained with DAPI (D, H, L, P and T).
To obtain further evidence for a Sox10-antagonizing role of Sox5, we cotransfected luciferase reporters under the control of the Dct or Mitf promoter in B16 melanoma cells with the Sox5- and Sox10-specific shRNA vectors. When luciferase activities were determined 2 days posttransfection, we observed a significantly reduced expression of both reporters in the presence of the Sox10-specific shRNA (Figure 10A and B). When the expression plasmids for the Sox5-specific shRNA or the scrambled control versions were cotransfected instead, activities of both Dct and Mitf promoters remained unaffected (Figure 10A and B and data not shown). Intriguingly, simultaneous transfection of equal amounts of these reporters in the presence of the Sox10-specific shRNA resulted in a significantly reduced expression of both reporters (Figure 10A and B), indicating a Sox10-antagonizing role of Sox5.

Figure 8. Sox5 and Sox10 influence melanocyte-specific gene expression in B16 melanoma cells. Immunocytochemistry was carried out on B16 melanoma cells transfected with an shRNA vector specific for Sox10 (A, B, E, F, I and J), the corresponding scrambled version (C, D, G, H, K and L), an shRNA vector for Sox5 (M, N, Q, R, U and V), its corresponding scrambled version (O, P, S, T, W and X) and a combination of Sox5- and Sox10-specific shRNA vectors (Y and Z). Immunocytochemistry was performed using antibodies against Sox10 (A–D, M–P), Sox5 (E–H, Q–T) and Mitf (I–L, U–Z) and immunolabeling is shown in red. Transfected cells were visualized by GFP autofluorescence (B, D, F, H, J, L, N, P, R, T, V, X and Z) (green color). Arrowheads indicate transfected cells in which expression of endogenous proteins is lost or significantly reduced.

Figure 9. The influence of Sox6 on melanocyte-specific gene expression in B16 melanoma cells. Immunocytochemistry was carried out on B16 melanoma cells transfected with an shRNA vector specific for Sox5 (A and B), for Sox6 (C–J) and a combination of Sox6- and Sox10-specific shRNA vectors (K and L). Immunocytochemistry was performed using antibodies against Sox6 (A–D), Sox5 (E and F), Sox10 (G and H) and Mitf (I–L) and Mitf (I–L) (in red). Transfected cells were visualized by GFP autofluorescence (B, D, F, H, J, L, N and P) (green color). Arrowheads indicate transfected cells in which expression of endogenous proteins is lost or significantly reduced.
of expression plasmids for Sox5- and Sox10-specific shRNA led to a statistically significant partial recovery of the activities of the Dct and the Mitf promoters when compared to transfections in which only the Sox10-specific shRNA was present. This argues that lowering of Sox5 amounts indeed helps to alleviate the effects of Sox10 reductions on melanocyte-specific gene expression.

Sox5 is bound in B16 cells to promoters of melanocytic Sox10 target genes and recruits transcriptional corepressors

We used chromatin immunoprecipitation assays to ask whether Sox5 was also bound to Sox10 target gene promoters in vivo. For this purpose, chromatin was prepared from B16 melanoma cells and precipitated with control antibodies or antibodies directed against Sox5 and Sox10 (Figure 10C). As expected, both Dct and Mitf promoters were specifically enriched in the precipitate obtained with the Sox10-specific antibody, but not in the precipitate from control IgG reactions. Both promoters were also preferentially immunoprecipitated from chromatin when Sox5-specific antibodies were used. In contrast to the promoter region, no enrichment was observed in the immunoprecipitations for distal upstream regions of the Dct and Mitf genes or the β-actin promoter.
proving specificity (Figure 10C). Chromatin immunoprecipitation thus confirmed that Sox5 as well as Sox10 occupy the Det and Mitf promoters in B16 melanoma cells.

Previous studies on Fgf3 gene regulation in the inner ear and cyclin D1 gene regulation in pancreatic β-cells had shown that Sox6 inhibits gene expression by recruiting the CtBP2 and HDAC1 corepressors to the respective promoters (50,51). To address whether the effects of the closely related Sox5 on melanocytic gene expression involved a similar mechanism, we first checked by chromatin immunoprecipitation whether these corepressors were also present on the Det and Mitf promoters. Indeed, we were able to precipitate higher amounts of both promoters from B16 chromatin with anti-HDAC1 and anti-CtBP2 antisera than with control IgG, whereas no enrichment was obtained in the HDAC1- or CtBP2-specific precipitates for control fragments from the distal upstream regions (Figure 10D).

 Luciferase reporter assays in transiently transfected Neuro2a cells furthermore showed that HDAC1 and CtBP2 on their own had no significant effect on Sox10-dependent activation of the Mitf promoter (Figure 10E). Both were, however, capable of enhancing the inhibitory effects of limiting amounts of the long Sox5 isoform on Sox10-dependent promoter activation, suggesting that recruitment of these corepressors to the Mitf promoter can indeed occur via Sox5. Such a conclusion was additionally strengthened by chromatin immunoprecipitation experiments on Neuro2a cells transiently transfected with Sox5, a HA-tagged version of CtBP2 or a combination of both, as enrichment of the Mitf promoter in chromatin precipitated with an antibody against the HA tag of CtBP2 was only observed when the long Sox5 isoform was simultaneously present in the transfected cell (Figure 10F).

**DISCUSSION**

Here, we have shown that Sox5 is expressed in the melanocyte lineage during mouse embryonic development. This extends previous findings on the occurrence of Sox5 in early neural crest cells (15) and identifies the melanocyte lineage in addition to peripheral glia as the second neural-crest-derived cell type in which Sox5 expression is maintained after the specification event. In the melanocyte lineage, Sox5 expression was restricted to melanoblasts. Sox5 was furthermore the major SoxD protein in melanoblasts where it occurred in its long isoform.

Analysis of c-Kit, Det and Mitf as three independent markers revealed a surprisingly normal generation of melanoblasts in Sox5-deficient mice. Considering the dominance of Sox5 expression over Sox6 or Sox13 expression in melanoblasts, it appears unlikely that a melanocytic phenotype would be completely obscured in Sox5-deficient mice by other SoxD proteins, although examples of coexpression and functional redundancy exist for Sox5 and Sox6 in the development of other cell lineages such as chondrocytes and oligodendrocytes (52,53).

As a consequence, single mouse mutants exhibited only mild chondrocytic and oligodendrocytic phenotypes, whereas the double mutant showed severe developmental defects (7,9).

A fairly normal generation of melanoblasts would be expected if Sox5 predominantly functioned as a modifier rather than a developmental regulator of its own. Sox5 has indeed been previously reported to modulate the function of the SoxE proteins Sox9 and Sox10. Sox5 enhances the function of Sox9 during several consecutive phases of chondrocyte development (44,54), but attenuates the effects of Sox9 on specification and of Sox10 on terminal differentiation during oligodendrocyte development (9). Sox5 also performs such a modulatory role in early development of the melanocyte lineage as indicated by the fact that the dramatically reduced generation of melanoblasts in Sox10 heterozygous embryos is partially restored by additional deletion of Sox5. As already observed in the oligodendrocyte lineage, Sox5 appears to counteract the activity of Sox10.

In contrast to melanoblasts of the early mouse embryo, B16 melanoma cells not only expressed Sox5 and Sox10, but also Sox6 and Sox9. The melanoma is thus more complicated than the melanoblast. Nevertheless, our results from shRNA experiments in B16 melanoma cell cultures were fully compatible with an antagonistic effect of Sox5 on Sox10 activity.

Previous studies had failed to detect any direct protein–protein interactions between Sox5 and Sox10 in solution (9). There is thus no evidence that Sox5 may function by sequestering Sox10 away from its target genes in inactive complexes. Instead, Sox5 was detected by chromatin immunoprecipitation on the same regulatory regions that have previously been shown to mediate Sox10 responsiveness to its melanocytic target genes (25–29,32–34). EMSA furthermore indicated that Sox5 binds to the same sequences that are Sox10 response elements. Because binding of Sox5 and Sox10 were mutually exclusive in vitro, it can be assumed that competition for binding sites may be one of the ways in which Sox5 interferes with the function of Sox10. However, it should be kept in mind that most of the Sox10-responsive promoters and enhancers contain multiple binding sites for this protein, which contribute to different extents to the overall rate of induction (55). This is also the case for the melanocytic Sox10 target genes Det and Mitf(25,28,29,32,34). Competition for binding to a particular site therefore does not exclude the possibility that both Sox5 and Sox10 are bound to different sites of the Det or Mitf promoter at the same time. Under these conditions, it is probably essential that Sox5 recruits the HDAC1 and CtBP2 transcriptional corepressors to the promoters of melanocytic Sox10 target genes as we have shown in this study. A similar capability has previously reported for Sox6 in its role as a repressor of Fgf3 gene expression in the inner ear, cyclin D1 gene expression in pancreatic β-cells and e-globin expression during definitive erythropoiesis (12,50,51). Corepressor recruitment may thus be a common feature of SoxD proteins.

Intriguingly, Sox5 has recently also been shown to recruit coactivators to Sox9 target genes in chondrocytes
and thereby enhance Sox9 function (56). With its ability to alternatively recruit corepressors or coactivators in different developmental contexts, the ability of Sox5 to modulate the activity of SoxE proteins is clearly mechanistically complex and likely to have multiple facets.

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