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Original Paper

Coexpression of SOX10/CD271 (p75^{NTR}) and β-Galactosidase in Large to Giant Congenital Melanocytic Nevi of Pediatric Patients

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Key Words

Neural crest · Congenital melanocytic nevi · Nevus · Stem cells · Melanoma · SOX10 · BRAF · NRAS

Abstract

Background: Congenital melanocytic nevi (CMNs) are melanocytic neoplasms that can transform into melanoma. However, this development is impeded in the majority of cases and mostly affects patients with large or giant CMNs. ***Methods:*** To elucidate mechanisms that keep CMNs from malignant transformation, CMN tissue biopsies were investigated for p-ERK and senescence markers by immunohistochemistry and for SOX10/CD271 (p75^{NTR}) by immunofluorescence. CMN cells were cultivated, and MTT assays were performed for evaluating cell viability. Mutation status for NRAS and BRAF was performed by real-time PCR. ***Results:*** 13 CMNs (from patients aged 0.5–11.8 years, mean: 2.7) showed immunoreactivity for SOX10/CD271 (p75^{NTR}) in 34.2%. p-ERK was immunoreactive in 80% (4/5); β-galactosidase was significantly stronger expressed in CMNs compared to melanocytic nevi of patients over 70 years ($p = 0.0085$). The 5 CMN cultures were immunoreactive for SOX10/CD271 (p75^{NTR}) in 36.7%. By silencing SOX10 by siRNA in 2 CMN cell cultures, cell viability decreased significantly. NRAS^{Q61K} mutation was found in 91.7% (11/12) and BRAF^{V600E} in 6.3% of all analyzable CMNs (1/16). ***Conclusions:*** Oncogene-induced senescence might prevent malignant transformation through activation of the mitogen-activated protein kinase pathway. SOX10 is necessary for the viability of human CMN cell cultures and may be responsible for clinical changes during aging.

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Introduction

Congenital melanocytic nevi (CMNs) are benign melanocytic tumors that exist at birth [1]. Their occurrence is caused by postzygotic mutations that result in a disruption of the melanocytic migration from the neural crest to the skin during embryonic development [2]; the earlier this event takes place, the larger the CMNs will develop in size [3].

CMNs share features with melanomas as they grow in diameter and also form secondary proliferations [4, 5]. The risk of developing melanomas rises with the size of the lesion [6] and is 2–3% in large and giant CMNs [7–9]. However, melanomas in patients with large or giant CMNs may develop outside the CMN in other structures [6, 7, 10–16].

Mutations in the NRAS gene are found in 70–81% of large and giant CMNs and in 80% of satellite nevi [17], while only 0–15% of the CMNs show a V600E mutation in the BRAF gene [18–20]. However, the role of the mitogen-activated protein kinase (MAPK) signaling cascade, which plays a crucial role in melanoma development [21], has not been extensively investigated for the CMNs so far. For noncongenital melanocytic nevi, activation of the downstream effector ERK has been found in 30–50% of dysplastic nevi while it is activated in 85% of melanomas [22–25].

Mechanisms that drive CMNs into malignancy are yet inconclusive. Shakhova et al. [26] have recently revealed the essential role of the transcription factor SOX10 for the formation of melanoma and the expression of SOX10 in CMNs. In this work, they suggest SOX10 to be a major driver for the genesis of melanomas and CMNs. Using the *Tyr::Nras*^{Q61K} *INK4a*$^{-/-}$ mouse model, Shakhova et al. could show that melanoma was induced by the NRAS^{Q61K} mutation in the melanocyte lineage. By silencing SOX10 in melanoma cells, cell proliferation and survival as well as tumor formation in vivo was suppressed. These findings propose that SOX10, which is important for neural crest development, is also involved in the development of melanoma and CMNs in mice. The question arises whether SOX10 is also required for the development of CMNs and melanoma in humans.

Therefore, to affirm the presence of neural crest markers in human CMNs of children and to explore possible mechanisms that might keep the CMNs from malignancy, we explored the expression of the neural crest stem cell marker SOX10/CD271 (p75^{NTR}), p-ERK and senescence markers in CMNs in comparison to other nevus types and melanoma.

Materials and Methods

Fresh tissue material of CMNs of children was received from the Department of Plastic and Reconstructive Surgery, Pediatric Hospital, University of Zurich, Switzerland. None of the children showed signs of leptomeningeal involvement of the CMNs, and none of them had developed melanoma so far. The 3 CMNs from older patients derived from the Department of Dermatology, University Hospital of Zurich, Switzerland, one of them (from a patient aged 74 years) with a melanoma developing within the lesion. All other nevi were excised for cosmetic reasons. Written informed consent from all patients and/or their parents, respectively, was obtained following the guidelines of the Ethics Committee (Specialized Cantonal Ethics Subcommittee for Specialties Zürich, No. 840) and the Declaration of Helsinki. All lesions were histologically confirmed.

For further immunohistochemical investigations, 8 nevi with a histopathological congenital pattern derived from 1 female and 5 male patients, 8 melanocytic nevi without a congenital pattern and 8 melanocytic nevi from patients over 70 years were further taken. Five tissue specimens of primary melanoma which arose within a melanocytic nevus and 5 melanoma metastases were used for additional control.

The BRAF and NRAS mutation status was determined by mutation-specific real-time PCR as previously described [27].

Immunohistochemistry and Immunofluorescence, Mutation Status

Tissue samples were fixed in 4% formaldehyde overnight and embedded in paraffin. Paraffin-embedded blocks were sectioned at 6-μm thickness and deparaffinized in xylene, hydrated with ethanol and rinsed in distilled water. Forty-minute citrate buffer incubation (10 mM citric acid, 0.05% Tween 20, pH 6) in a steamer was used for epitope retrieval. Afterwards, sections were cooled for 20 min and rinsed in phosphate-buffered saline. The following primary antibodies were used during the 0.5-hour staining at room temperature: MelanA (Leica Microsystems, 1:60), HMB45 (Dako, 2:1), MITF (Dako, clone D5, 1:60), p16 (B+D, 1:100), KI67 (Dako, 1:3), β-galactosidase (ICN Pharmaceuticals; 1:100) and p-ERK [Cell Signaling, Phospho-p44/42 (ERK1/2), 1:200]. Chromogenic detection was performed using the alkaline phosphatase-antialkaline phosphatase method except for p-ERK for which the phosphatase-antialkaline phosphatase method was used. For p-ERK, mammary carcinoma served as controls; for CD271 (p75^{NTR}) and SOX10 control, brain tissue was used.

Semiquantitative assessment was performed for stain intensity (0 = none; 3 = strong) and percentage of reactive cells (0–100%); the product of both was taken (grade × percentage).

For immunofluorescent double-staining against SOX10/CD271 (p75^{NTR}), slides were preincubated with bovine serum albumin 1% for 30 min and incubated with primary antibodies [SOX10: R&D; CD271 (p75^{NTR}): Alomone lab., each 1:400] overnight at 4°C, followed by the incubation with Cy3-conjugated anti-mouse and Alexa488-conjugated anti-rabbit secondary antibodies for 1 h at room temperature (Jackson ImmunoResearch Laboratories, and Invitrogen, each 1:500). Nuclei were counterstained with Hoechst 33342 solution (Invitrogen, 1:10,000). Staining only with secondary antibodies was used to evaluate background fluorescence and unspecific binding. Analysis was performed semiquantitatively by visual examination with an Olympus BX51TF. SOX10/CD271 (p75^{NTR}) double-reactivity was evaluated using an upright fluorescence microscope and upright confocal microscope (AF6000 and SP2, Leica Microsystems) at ×40 magnifications. Photographs were taken using a spectral detection camera on the system. Positivity was assessed when cells were double-positive for nuclear SOX10 and plasma membrane CD271 (p75^{NTR}) staining; histological co-localization of positive subpopulation was evaluated. Hair follicles within the tissue served as a positive control; for a negative control, Merkel cell carcinoma [Mangana J. et al., unpublished data] was taken. Imaging and analysis was performed using the integrated Leica Software.

Cell Cultures

Fresh tissues of 4 large to giant CMNs of children and 1 of a 75-year-old patient served for the establishment of cell cultures. Cells were released from tissue sections, and cultured as adherent cells for 10–14 passages using melanocyte growth medium (Melanocyte Growth Medium M2, PromoCell). Another portion was cultured for nonadherent spheres in D-MEM/F-12 (1:1) medium [supplemented with B27 stem cell culture supplement without vitamin A, basic fibroblast growth factor 2, epidermal growth factor, penicillin, streptomycin, Fungizone, and human leukemia-inhibiting factor in hydrophobic poly-HEMA-coated flasks for up to 4 passages after culturing cells as adherent cells for 6–8 passages (all Invitrogen Corporation)].

Immunocytological detection of MelanA, and SOX10/CD271 (p75^{NTR}), was performed with cell pellets containing 105 cells, resuspended in 4% formalin, fixed for 6 h at 4°C, and mixed in 1.5% NuSieve low-melting agarose (FMC Biopractice). After cooling, suspension was injected into chambers and dehydrated in ethanol and xylene. Blocks of paraffin-embedded cells were sectioned at 6-μm-thick slides for staining as described above. For anti-β-

galactosidase detection cytospins using 2 × 104 trypsinized and washed cells were made. Cells were centrifuged onto a SHANDON glass and fixed in acetone. Staining was performed as described above.

MTT Assay and siRNA Knockdown

Cell viability was measured by the MTT assay for the two CMN cell cultures (ZM07 and SC06) as previously described [28, 29]. Silencing RNA (siRNA) transfection of CMN cell cultures was carried out using INTERFERin transfection solution according to the manufacturer's protocol (Polyplus-transfection, Illkirch, France). Cells were transfected with 10 nM of siRNA (Qiagen) for 72 h before RNA was extracted. As control siRNA, the All-Star negative siRNA sequence (Qiagen) was used, and gene-specific siRNAs targeting siSOX10 (SI00729414, SI00729421) were obtained from Qiagen.

Statistics

Statistical analyses were conducted with GraphPad 5 (San Diego, Calif., USA) for Windows. A p value of less than 0.05 was considered as statistically significant. For evaluating the p values in the immunohistochemistry section, unpaired t test was used. Standard deviation (SD) and 95% confidence intervals (CI) were computed.

Results

Demographics and Mutation Status

Thirteen CMNs (all present at birth) were derived from 5 girls and 5 boys with a median age of 1.2 years (0.5–11.8 years) and had a median maximal diameter of 7 cm (2–30 cm). Comprehensive patient demographics are illustrated in online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000362490).

The NRAS^{Q61K} mutation was found in 91.7% of all analyzable CMNs (11/12); in 4 cases, data were not available, most likely due to low DNA quality. The BRAF^{V600E} mutation was found in 1 of 16 CMNs, and 15 CMNs were wild type for this mutation (online suppl. table 1).

Immunohistochemical and Immunofluorescence Analysis

p-ERK and MITF

The p-ERK expression in most CMNs was stronger in the upper dermis with weaker staining towards deeper structures (data not shown). Remarkably, CMNs that showed a deep infiltration demonstrated an inverse pattern with more immunoreactivity in deeper structures than in the upper parts of the dermis (fig. 1a, b). Hair follicles and blood vessels were strongly immunoreactive in both groups (fig. 1b). Interestingly, within each melanoma metastasis, p-ERK was unequally expressed in different, sharply demarcated areas (fig. 1e).

Four of 5 CMNs (80%) showed immunoreactivity to p-ERK while 100% of the primary melanoma and melanoma metastases were immunoreactive (each 5/5) (fig. 1c–g). The nevi around the primary melanoma showed a trend for less immunoreactivity to p-ERK than the primary melanoma within the same nevus although it was not significant ($p = 0.0856$). p-ERK immunoreactivity in melanoma metastasis was significantly stronger than in melanocytic nevi ($p < 0.0001$), CMN ($p = 0.0052$), primary melanoma ($p = 0.0388$) and the associated melanocytic nevus ($p = 0.0017$) (fig. 1g).

MITF expression did not differ within the various nevus groups (i.e. CMN, noncongenital melanocytic nevi with a congenital pattern, melanocytic nevi of patients over 70 years, other melanocytic nevi).

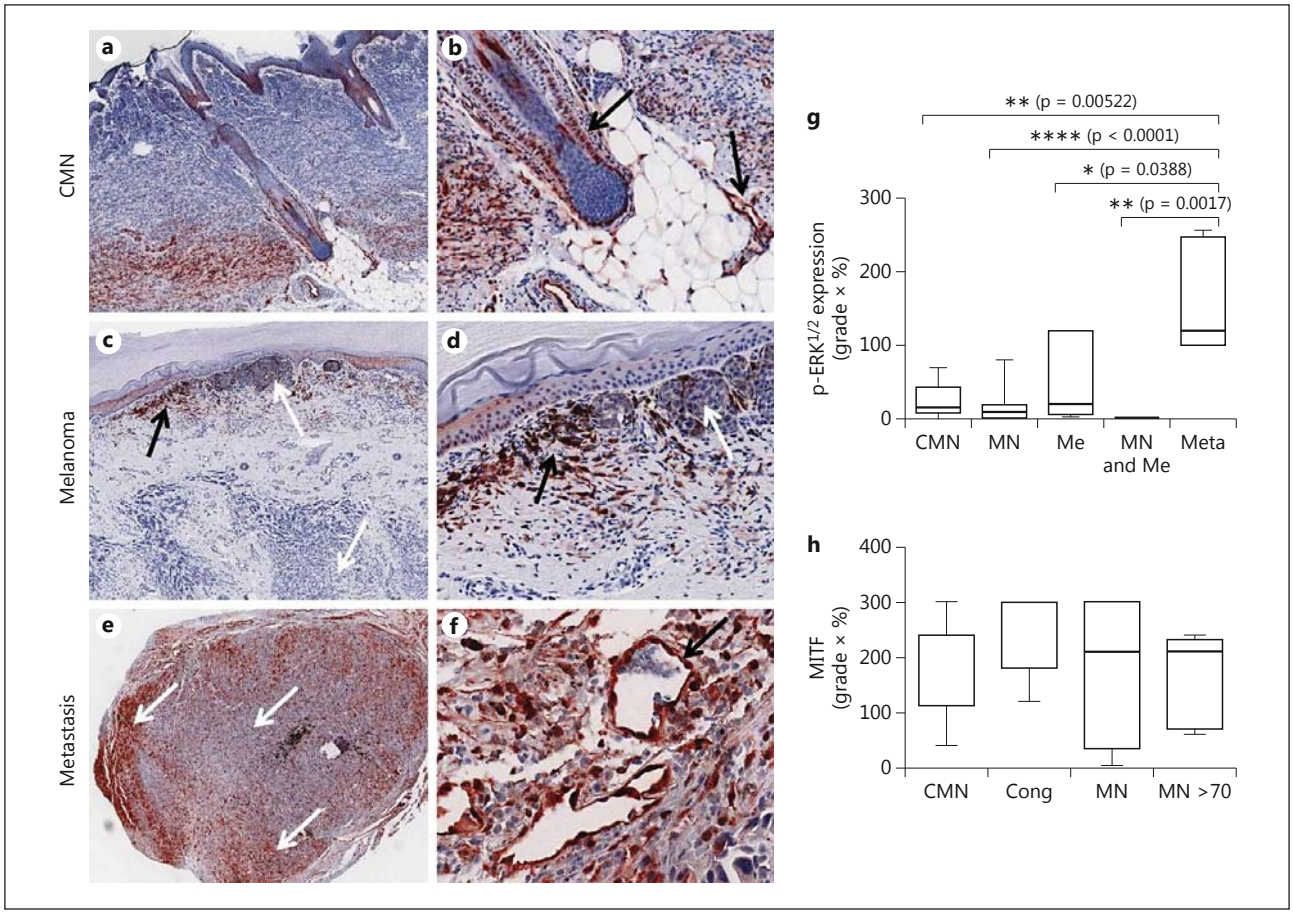

Fig. 1. Immunohistochemistry for p-ERK in CMNs (**a**, ×10 magnification; **b**, ×20 magnification), primary melanoma arising within a melanocytic nevus (**c**, ×10 magnification; **d**, ×20 magnification, black arrows for melanoma, white arrows for nevus), and melanoma metastasis (**e**, ×10 magnification; **f**, ×40 magnification). The strong immunoreactivity in the blood vessels and hair follicles is indicated by the black arrows. White arrows indicate areas of strong and slight immunoreactivity, respectively, in the melanoma metastasis. **g** Immunoreactivity in inner hair cells by boxplots. Note the significant difference of expression between melanoma metastases and CMN, melanocytic nevus, nevus-associated primary melanoma and the nevus around the melanoma. **h** Boxplots of MITF expression between the different nevus types. Notice the lack of difference between all nevus groups. MN = Melanocytic nevus; Me = melanoma; MN and Me = nevus around the melanoma; Meta = melanoma metastases; Cong = melanocytic nevus with a congenital pattern; MN >70 = melanocytic nevus of patients over 70 years.

Senescence and Proliferation

CMNs of the children were significantly more immunoreactive to β-galactosidase than melanocytic nevi of patients over 70 years ($p = 0.0085$; fig. 2o). Except for the hair follicles, which strongly expressed β-galactosidase, β-galactosidase expression was predominantly found in the junctional areas rather than in the deeper dermis (fig. 2d, h). However, CMNs which showed a deep dermal infiltration also showed stronger immunoreactivity in the deeper dermal areas (fig. 2l). β-Galactosidase-positive areas were also strongly immunoreactive for p16 but not for the proliferation marker KI67. As expected, CMNs were significantly less immunoreactive to the proliferation marker KI67 compared to primary melanoma ($p \leq 0.018$) or melanoma metastasis ($p = 0.0081$) (online suppl. fig. 1).

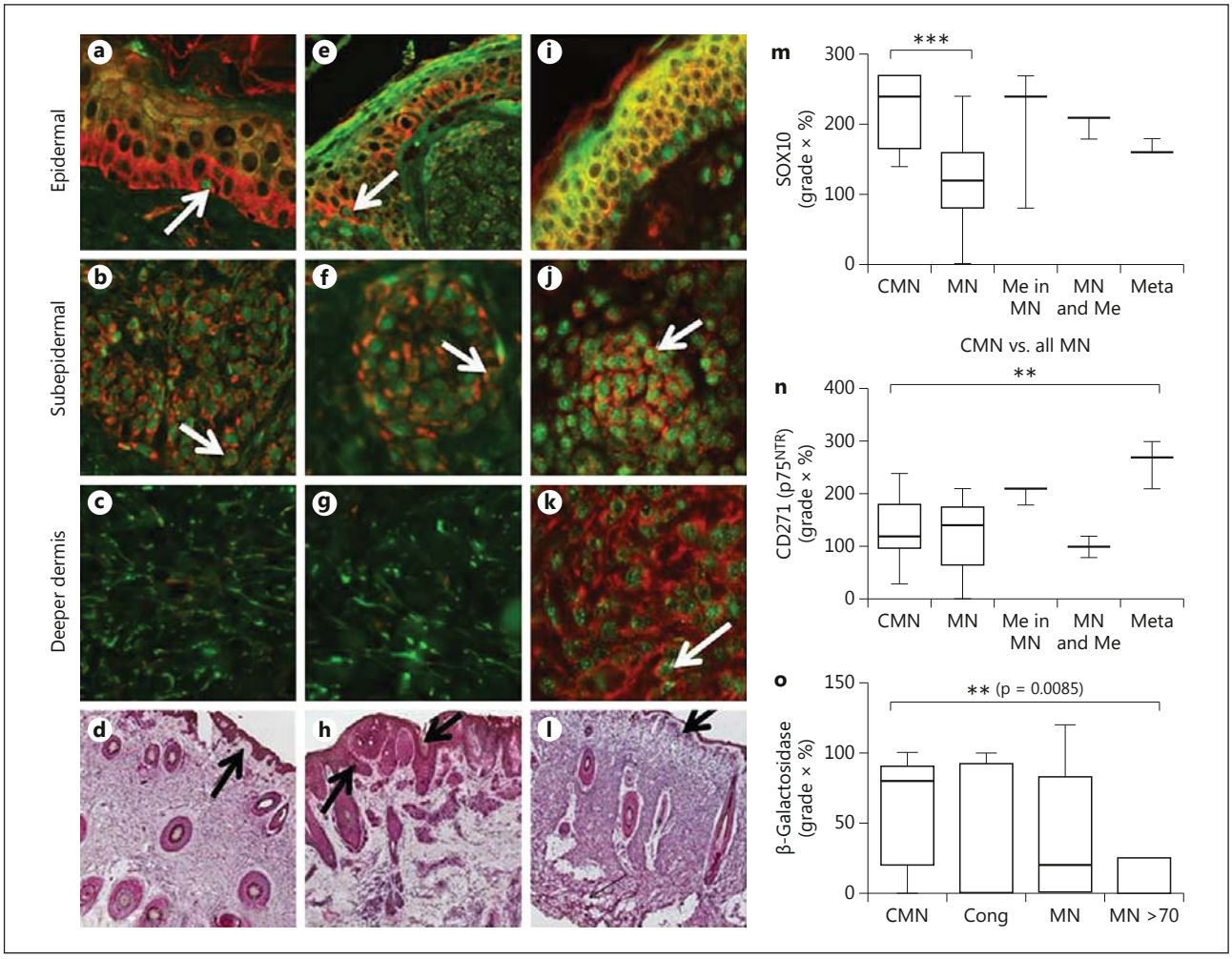

Fig. 2. SOX10/CD271 (p75^{NTR}) double-staining and anti-β-galactosidase staining of a compound type CMN deriving from a 3.7-year-old (**a–d**) and 1.9-year-old (**e–h**) child, respectively, and a CMN with deep dermal infiltration derived from a 0.8-year-old child (**i–l**). SOX10/CD271 (p75^{NTR}) double-positivity is found in a few cells in the epidermal layer and in nests of the upper dermis (**b**, **f**, **j**). Deeper dermis is free of double-positive SOX10/CD271 (p75^{NTR}) expressing cells in compound types (**c**, **g**) but strongly positive in the deep dermal infiltrating CMN (**k**). **d**, **h**, **l** Corresponding β-galactosidase staining. **m–o** Boxplots for SOX10 (**m**), CD271 (p75^{NTR}) (**n**) and β-galactosidase (**o**). CMNs showed significantly stronger SOX10 expression than other nevi (p = 0.0027) (**m**) and less expression of CD271 (p75^{NTR}) than melanoma metastases (p = 0.0042) (**n**). CD271 (p75^{NTR}) was significantly stronger in primary melanoma associated with a melanocytic nevus than the surrounding nevus (p = 0.00028) (**n**). β-Galactosidase was significantly stronger expressed in congenital nevi compared to nevi of patients over 70 years of age (p = 0.085) (**o**). MN = Melanocytic nevus; Me = melanoma; MN and Me = nevus around the melanoma; Meta = melanoma metastases; Cong = melanocytic nevus with a congenital pattern; MN >70 = melanocytic nevus of patients over 70 years. Red = CD271 (p75^{NTR}); green = SOX10; white arrows = double-positive cells.

Neural Crest Stem Cell Markers

Immunofluorescent double-staining for SOX10/CD271 (p75^{NTR}) on 12 samples showed double-positivity in 34.2% of the cells of each CMN specimen (95% CI = 0–70; SD = 22.45) except for the older patient of 18.5 years in whom no double-positivity was observed. In the other CMNs, expression was stronger in the upper dermis and continuously fainter towards

the deeper structures (fig. 2a–l). Adnexal structures such as hair follicles and blood vessels strongly expressed SOX10 and CD271 (p75^{NTR}) (online suppl. table 2; fig. 2a–l).

Immunohistochemistry showed significantly stronger immunoreactivity of SOX10 in CMNs of children compared to noncongenital melanocytic nevi (p = 0.0061) and nevi of patients over 70 years (p = 0.0035). There was no significant difference of SOX10 expression between nevus-associated primary melanoma, the surrounding nevus or melanoma metastasis (fig. 2m).

CD271 (p75^{NTR}) expression in CMNs showed a trend for less immunoreactivity compared to primary melanoma (p = 0.1325) but significant stronger expression in melanoma metastases (p = 0.0057). Immunoreactivity to CD271 (p75^{NTR}) was significantly stronger in primary melanoma associated with a melanocytic nevus than the surrounding nevus (p = 0.00028) (fig. 2n).

Morphology and Characteristics of Cultured Cells (MTT Assay)

CMN cells in cultures showed a robust behavior in terms of the ability for cultivation for several passages. Two of the CMN cell cultures were fast proliferating (i.e. ZM07: 9 passages were run through; FE08: 18 passages), one intermediate (SC06: 12 passages) and one slowly (LJ08: 8 passages) but could be kept in culture. One cell culture derived from the CMN of the 74-year-old patient in which a primary melanoma arose within (SW34, only CMN material was taken, 12 passages). Notably, in the tissue which served for cell cultivation of the latter CMN, melanoma was histologically and immunohistochemically excluded.

Freezing and defreezing for reculturing did not harm the cells. Notably, nevus cells changed their morphology after culturing over several weeks. At early passages, the cells showed a large body with only short dendrites, whereas over time cells extended the length of their dendrites while their body maintained the same morphology or even became smaller (fig. 3). Due to a slower proliferation rate after several weeks, the cells did not cluster as much as at the beginning. The granules and the pigment within their bodies, however, remained at the same level. Overall, the CMN cells derived from children survived through 9–18 passages, and the cells from the 74-years-old patient continued growing through 12 passages.

66.7% of the cultured CMN cells were immunoreactive for MelanA (95% CI = 30–90; SD = 32.2) and 100% were reactive for HMB45 (SD = 0). The cells showed double-reactivity for SOX10/CD271 (p75^{NTR}) in 36.7% (95% CI = 10–60; SD = 25.2) of the cells of the children's CMNs but only in 10% of the cells of the 74-year-old patient. In children's CMNs, the cells were immunoreactive to β-galactosidase in 43.3% (95% CI = 30–50; SD = 11.6), to p16 in 41.7% (95% CI = 30–50; SD = 10.4), and to KI67 in 31.0% (95% CI = 2–60; SD = 41) (fig. 3).

By knocking down SOX10 with 2 independent siRNAs in 2 different CMN cultures (ZM07 and CS06), significant downregulation of SOX10 expression was observed (fig. 4a, b). Significant loss of cell viability in both CMN cultures was further investigated by the MTT assay with one of the cultures exhibiting an almost 50% reduction in viability as a result of SOX10 knockdown (ZM07; fig. 4c, d).

Discussion

There is no clear understanding of how CMNs develop – which are benign melanocytic neoplasms that are derived from the neural crest – and which mechanisms keep them from malignant transformation. So far, activating mutations of the MAPK kinase pathway have been suggested to play a role [17, 19]. Indeed, depending on the size, 2–3% of the large CMNs develop into melanomas [7–9, 30].

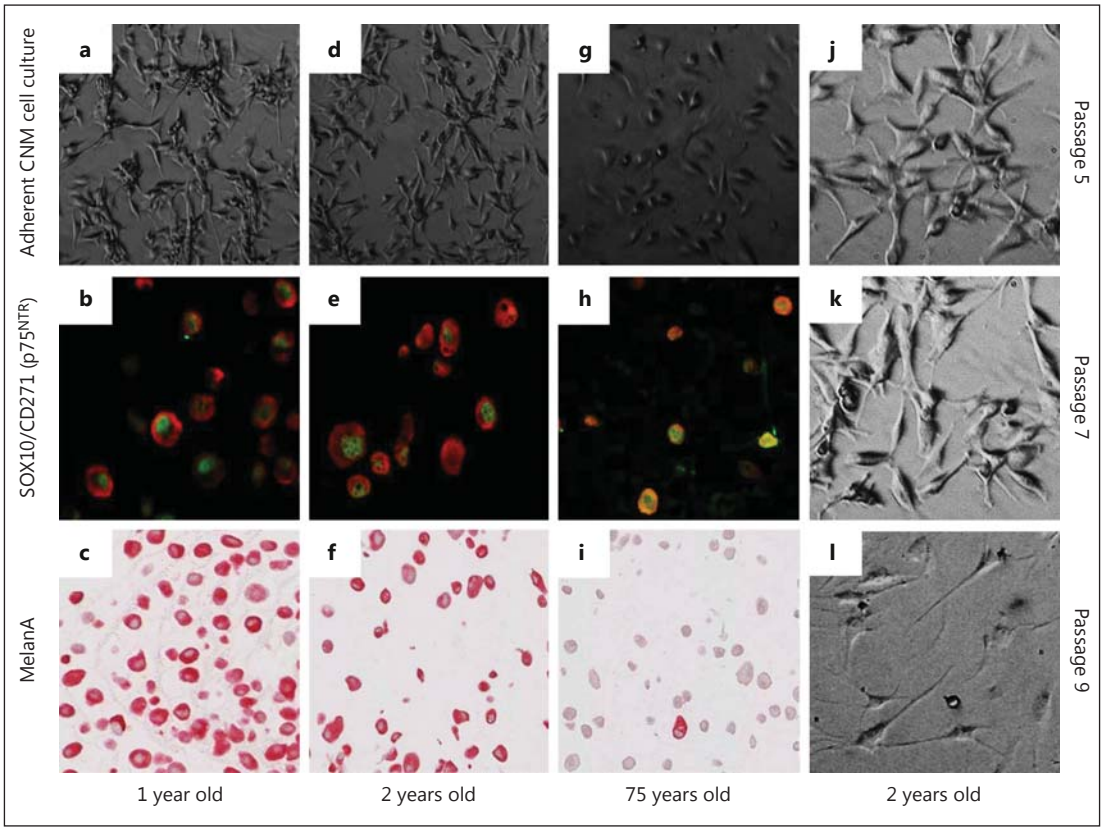

Fig. 3. Adherent CMN cell cultures in phase contrast microscopy (**a**, **d**, **g**) with corresponding anti-SOX10/CD271 ($p75^{NTR}$) (**b**, **e**, **h**) anti-MelanA (**c**, **f**, **i**) staining of 1- and 2-year-old children (**a–c** and **d–f**), respectively, and of the 75-year-old patient (**g–i**). About 30% of the cells show immunoreactivity to SOX10/CD271 ($p75^{NTR}$) double-stain in the first two cultures, while the third culture reveals reactivity in about 10% of the cells. Cells of the first two cultures are all positive for anti-MelanA staining; the third culture is negative in the majority. **j–l** Cell cultures of different passages of the 1.6-year-old girl (ZM07): passage 5 (**j**), passage 7 (**k**) and passage 9 (**l**). Note the elongation of the dendrites while the size of the body stays constant, and clustering of the cells decreases.

In the MAPK pathway, ERK is downstream of activating NRAS and BRAF mutations, and ERK phosphorylation indicates pathway activation. Unlike Jorgensen et al. [23], who did not find p-ERK immunoreactivity in nevi, and according to Oba et al. [31], who found 33% of nevus cells to be immunoreactive, we detected immunoreactive cells in 80% of the melanocytic nevi (20/25) and 100% of the malignant melanocytic lesions (10/10). However, melanocytic nevi were less immunoreactive to p-ERK than primary melanoma or melanoma metastases ($p = 0.04$). These findings suggest that the majority of melanocytic nevi also harbor activating mutations in the MAPK pathway, although significantly less than in melanoma metastases. Within each melanoma metastasis, p-ERK was heterogeneously expressed in different areas indicating diverse subpopulations that may reside within one single metastasis [32]. Notably, most blood vessels expressed strong immunoreactivity to p-ERK. This observation is consistent with clinical findings that melanoma patients under MEK inhibitors suffer consistently from edematous skin changes [33].

Loss of senescence is suspected to facilitate cellular malignancy [34]. Several studies investigated the existence of senescence in CMNs of children with controversial results most

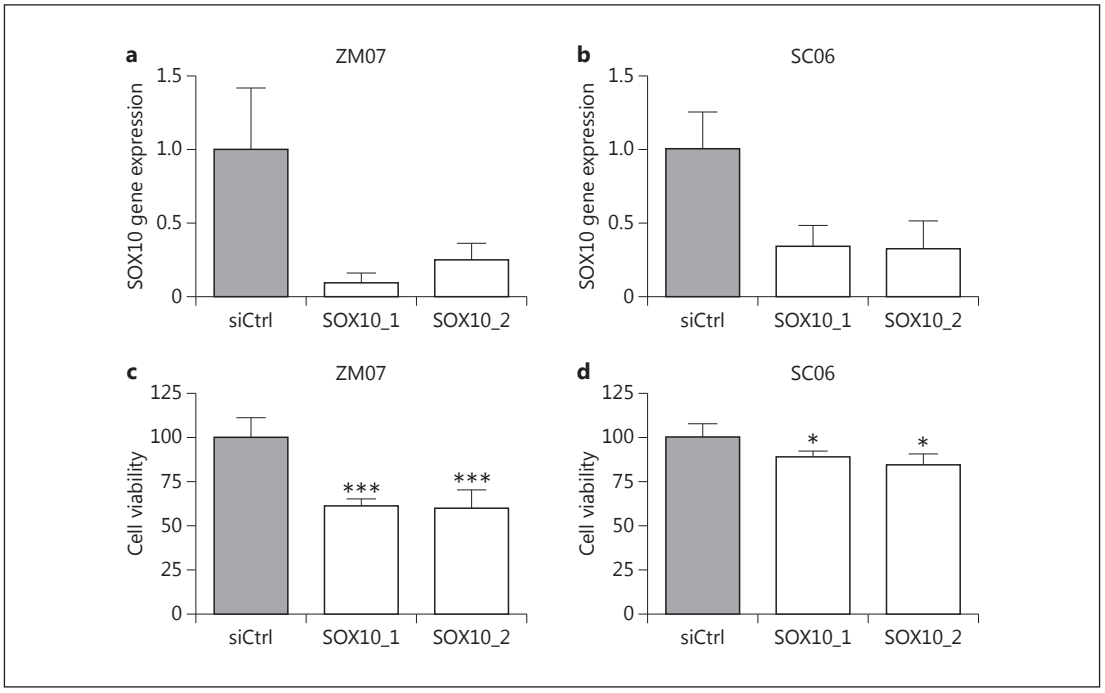

Fig. 4. The results of MTT assays in cell lines ZM07 (**a**, **c**) and SC06 (**b**, **d**) upon SOX10 knockdown. SOX10 mRNA expression was knocked down by two independent siRNAs for CMN cell cultures ZM07 (**a**) and SC06 (**b**). Cell viability was significantly reduced in both CMN cultures with a much stronger effect seen in the cell culture ZM07 with 60% viability (**c**) compared to SC06 with 85% viability (**d**) (* $p < 0.05$; *** $p < 0.001$).

likely due to inconsistent techniques [35–37]. Oncogene-induced senescence in melanocytic nevi due to activated oncogenes such as BRAF has been suggested earlier [20, 38]. However, CMNs usually do not harbor BRAF mutations, as previously described and also shown by our results in which only 1 of 16 CMNs was mutated in the BRAF locus. Nevertheless, $NRAS^{Q61K}$ mutations are prevalent in CMNS as also shown by our samples, with the known activating mutations in 91.7% of all analyzable CMNs (11/12, 4 were not definable) [17, 19].

We found a significantly higher expression of the senescence marker β-galactosidase in CMNs than in melanocytic nevi of patients over 70 years of age. β-Galactosidase in CMNs was co-localized with p16 while the proliferation marker KI67 was absent in these areas. This strongly indicates the presence of senescence in these areas [35, 39].

The transcription factor SOX10 is expressed in neural crest stem cells. It is required for maintaining multipotency but also for maintenance of differentiated cells [40]. Primary melanoma and melanoma metastases show double-positivity for the neural crest stem cell marker SOX10/CD271 ($p75^{NTR}$) in sphere-growing cell cultures [41]. A mouse model with the $NRAS^{Q61K}$ mutation under the control of a tyrosine promoter and with a deletion of the cell cycle control gene INK4a has largely pigmented from birth that histopathologically mimics features of CMNs. SOX10 haploinsufficiency abrogated the $NRAS^{Q61K}$-induced hyperpigmentation and also prevented melanoma formation [26].

In our study, we show that human CMN cells are able to be cultured over many passages with good growth ability, showing heterogeneous morphology. SOX10/CD271 ($p75^{NTR}$) immunofluorescence analysis performed on cell cultures revealed double-reactivity in 36.7% of the CMN cells derived from 2 children below the age of 2 years and was stronger compared to the one of the 74-years-old patient. These findings suggest neural crest-like features with

stem cell-like properties in the CMNs of young patients. As observed in the tissues of the older patients with CMN, this property can be lost during aging. This may explain the clinical changes of CMNs with proliferation and growth during time [42, 43]. Furthermore, by showing significant loss of cell viability by knocking down SOX10 in CMN cell cultures, we show that SOX10 is necessary for viability of CMNs as it has been shown for human melanoma cells and in the NRAS mouse model [26].

Interestingly, areas in the CMNs that were immunoreactive for the senescence markers were also immunoreactive for SOX10/CD271 and p-ERK, including adnexal structures. Most of these cases even showed activated NRAS^{Q61K} mutations. These findings strongly suggest the existence of oncogene-induced senescence (by NRAS^{Q61K} mutation) in the CMNs and might be an underlying feature that prevents CMNs from malignant transformation despite an activated MAPK pathway.

A limitation of this study is the small sample numbers. Moreover, broader analysis on protein levels and investigation of the dedifferentiation ability of the cells would help validate our observations. However, despite these limitations, the presented work might have clinical implications in terms of treatment and prevention. We show that SOX10 plays a role in the maintenance of human CMNs, as in melanoma. Hence, by targeting SOX10, CMNs might be treated or even prevented.

In conclusion, through NRAS^{Q61K} activation, oncogene-induced senescence might protect CMNs from malignant transformation, although the MAPK pathway is activated. Concomitant expression of CD271 (p75^{NTR}) and SOX10 in human CMN tissue and cell cultures might account for the proliferation and survival of CMNs over time but without malignancy.

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Disclosure Statement

The authors state no conflict of interest. Maria B. Karpova now (after the evaluation of the study) is employed at F. Hoffmann-La Roche Ltd. However, there was no financial, equity, patenting or other relevant relationship or arrangement with F. Hoffmann-La Roche Ltd.

References

1 Kopf AW, Levine LJ, Rigel DS, Friedman RJ, Levenstein M: Prevalence of congenital-nevus-like nevi, nevi spili, and cafe au lait spots. Arch Dermatol 1985;121:766–769.
2 Grichnik JM, Ali WN, Burch JA, Byers JD, Garcia CA, Clark RE, Shea CR: Kit expression reveals a population of precursor melanocytes in human skin. J Invest Dermatol 1996;106:967–971.
3 Krengel S: Nevogenesis – new thoughts regarding a classical problem. Am J Dermatopathol 2005;27:456–465.
4 Kopf AW, Bart RS, Hennessey P: Congenital nevocytic nevi and malignant melanomas. J Am Acad Dermatol 1979;1:123–130.
5 Ruiz-Maldonado R: Measuring congenital melanocytic nevi. Pediatr Dermatol 2004;21:178–179.
6 Kinsler VA, Birley J, Atherton DJ: Great Ormond Street Hospital for Children Registry for congenital melanocytic naevi: prospective study 1988–2007. 1. Epidemiology, phenotype and outcomes. Br J Dermatol 2009;160:143–150.

7 Krengel S, Hauschild A, Schafer T: Melanoma risk in congenital melanocytic naevi: a systematic review. Br J Dermatol 2006;155:1–8.
8 Vourc'h-Jourdain M, Martin L, Barbarot S: Large congenital melanocytic nevi: therapeutic management and melanoma risk: a systematic review. J Am Acad Dermatol 2013;68:493–498.
9 Watt AJ, Kotsis SV, Chung KC: Risk of melanoma arising in large congenital melanocytic nevi: a systematic review. Plast Reconstr Surg 2004;113:1968–1974.
10 Swerdlow AJ, English JS, Qiao Z: The risk of melanoma in patients with congenital nevi: a cohort study. J Am Acad Dermatol 1995;32:595–599.
11 Bittencourt FV, Marghoob AA, Kopf AW, Koenig KL, Bart RS: Large congenital melanocytic nevi and the risk for development of malignant melanoma and neurocutaneous melanocytosis. Pediatrics 2000;106:736–741.
12 DeDavid M, Orlow SJ, Provost N, Marghoob AA, Rao BK, Huang CL, Wasti Q, Kopf AW, Bart RS: A study of large congenital melanocytic nevi and associated malignant melanomas: review of cases in the New York University Registry and the world literature. J Am Acad Dermatol 1997;36:409–416.
13 Hale EK, Stein J, Ben-Porat L, Panageas KS, Eichenbaum MS, Marghoob AA, Osman I, Kopf AW, Polsky D: Association of melanoma and neurocutaneous melanocytosis with large congenital melanocytic naevi – results from the NYU-LCMN registry. Br J Dermatol 2005;152:512–517.
14 Bett BJ: Large or multiple congenital melanocytic nevi: occurrence of cutaneous melanoma in 1,008 persons. J Am Acad Dermatol 2005;52:793–797.
15 Kinsler VA, Chong WK, Aylett SE, Atherton DJ: Complications of congenital melanocytic naevi in children: analysis of 16 years' experience and clinical practice. Br J Dermatol 2008;159:907–914.
16 Egan CL, Oliveria SA, Elenitsas R, Hanson J, Halpern AC: Cutaneous melanoma risk and phenotypic changes in large congenital nevi: a follow-up study of 46 patients. J Am Acad Dermatol 1998;39:923–932.
17 Kinsler VA, Thomas AC, Ishida M, Bulstrode NW, Loughlin S, Hing S, Chalker J, McKenzie K, Abu-Amero S, Slater O, Chanudet E, Palmer R, Morrogh D, Stanier P, Healy E, Sebire NJ, Moore GE: Multiple congenital melanocytic nevi and neurocutaneous melanosis are caused by postzygotic mutations in codon 61 of NRAS. J Invest Dermatol 2013;133:2229–2236.
18 Dessars B, De Raeve LE, Morandini R, Lefort A, El Housni H, Ghanem GE, Van den Eynde BJ, Ma W, Roseeuw D, Vassart G, Libert F, Heimann P: Genotypic and gene expression studies in congenital melanocytic nevi: insight into initial steps of melanotumorigenesis. J Invest Dermatol 2009;129:139–147.
19 Bauer J, Curtin JA, Pinkel D, Bastian BC: Congenital melanocytic nevi frequently harbor NRAS mutations but no BRAF mutations. J Invest Dermatol 2007;127:179–182.
20 Michaloglou C, Vredeveld LC, Mooi WJ, Peeper DS: BRAF(E600) in benign and malignant human tumours. Oncogene 2008;27:877–895.
21 Satyamoorthy K, Li G, Gerrero MR, Brose MS, Volpe P, Weber BL, Van Belle P, Elder DE, Herlyn M: Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. Cancer Res 2003;63:756–759.
22 Uribe P, Andrade L, Gonzalez S: Lack of association between BRAF mutation and MAPK ERK activation in melanocytic nevi. J Invest Dermatol 2006;126:161–166.
23 Jorgensen K, Holm R, Maelandsmo GM, Florenes VA: Expression of activated extracellular signal-regulated kinases 1/2 in malignant melanomas: relationship with clinical outcome. Clin Cancer Res 2003;9:5325–5331.
24 Yazdi AS, Ghoreschi K, Sander CA, Rocken M: Activation of the mitogen-activated protein kinase pathway in malignant melanoma can occur independently of the BRAF T1799A mutation. Eur J Dermatol 2010;20:575–579.
25 Zhuang L, Lee CS, Scolyer RA, McCarthy SW, Palmer AA, Zhang XD, Thompson JF, Bron LP, Hersey P: Activation of the extracellular signal regulated kinase (ERK) pathway in human melanoma. J Clin Pathol 2005;58:1163–1169.
26 Shakhova O, Zingg D, Schaefer SM, Hari L, Civenni G, Blunschi J, Claudinot S, Okoniewski M, Beermann F, Mihic-Probst D, Moch H, Wegner M, Dummer R, Barrandon Y, Cinelli P, Sommer L: Sox10 promotes the formation and maintenance of giant congenital naevi and melanoma. Nat Cell Biol 2012;14:882–890.
27 Schoenewolf NL, Dummer R, Mihic-Probst D, Moch H, Simcock M, Ochsenbein A, Gillessen S, Schraml P, von Moos R: Detecting BRAF mutations in formalin-fixed melanoma: experiences with two state-of-the-art techniques. Case Rep Oncol 2012;5:280–289.
28 Zipser MC, Eichhoff OM, Widmer DS, Schlegel NC, Schoenewolf NL, Stuart D, Liu W, Gardner H, Smith PD, Nuciforo P, Dummer R, Hoek KS: A proliferative melanoma cell phenotype is responsive to RAF/MEK inhibition independent of BRAF mutation status. Pigment Cell Melanoma Res 2011;24:326–333.
29 Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
30 Kaplan EN: The risk of malignancy in large congenital nevi. Plast Reconstr Surg 1974;53:421–428.
31 Oba J, Nakahara T, Abe T, Hagihara A, Moroi Y, Furue M: Expression of c-Kit, p-ERK and cyclin D1 in malignant melanoma: an immunohistochemical study and analysis of prognostic value. J Dermatol Sci 2011;62:116–123.
32 Civenni G, Walter A, Kobert N, Mihic-Probst D, Zipser M, Belloni B, Seifert B, Moch H, Dummer R, van den Broek M, Sommer L: Human CD271-positive melanoma stem cells associated with metastasis establish tumor heterogeneity and long-term growth. Cancer Res 2011;71:3098–3109.

33 Flaherty KT, Robert C, Hersey P, Nathan P, Garbe C, Milhem M, Demidov LV, Hassel JC, Rutkowski P, Mohr P, Dummer R, Trefzer U, Larkin JM, Utikal J, Dreno B, Nyakas M, Middleton MR, Becker JC, Casey M, Sherman LJ, Wu FS, Ouellet D, Martin AM, Patel K, Schadendorf D: Improved survival with MEK inhibition in BRAF-mutated melanoma. N Engl J Med 2012;367:107–114.
34 Campisi J: Suppressing cancer: the importance of being senescent. Science 2005;309:886–887.
35 Gray-Schopfer VC, Cheong SC, Chong H, Chow J, Moss T, Abdel-Malek ZA, Marais R, Wynford-Thomas D, Bennett DC: Cellular senescence in naevi and immortalisation in melanoma: a role for p16? Br J Cancer 2006; 95:496–505.
36 Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ, Peeper DS: BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature 2005;436: 720–724.
37 Cotter MA, Florell SR, Leachman SA, Grossman D: Absence of senescence-associated beta-galactosidase activity in human melanocytic nevi in vivo. J Invest Dermatol 2007;127:2469–2471.
38 Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR: Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. Cell 2008;132:363–374.
39 Michaloglou C, Soengas MS, Mooi WJ, Peeper DS: Comment on 'Absence of senescence-associated beta-galactosidase activity in human melanocytic nevi in vivo'. J Invest Dermatol 2008;128:1582–1583;author reply 1583–1584.
40 Harris ML, Buac K, Shakhova O, Hakami RM, Wegner M, Sommer L, Pavan WJ: A dual role for SOX10 in the maintenance of the postnatal melanocyte lineage and the differentiation of melanocyte stem cell progenitors. PLOS Genet 2013;9:e1003644.
41 Wong CE, Paratore C, Dours-Zimmermann MT, Rochat A, Pietri T, Suter U, Zimmermann DR, Dufour S, Thiery JP, Meijer D, Beermann F, Barrandon Y, Sommer L: Neural crest-derived cells with stem cell features can be traced back to multiple lineages in the adult skin. J Cell Biol 2006;175:1005–1015.
42 Strauss RM, Newton Bishop JA: Spontaneous involution of congenital melanocytic nevi of the scalp. J Am Acad Dermatol 2008;58:508–511.
43 Vilarrasa E, Baselga E, Rincon C, Alomar A: Histologic persistence of a congenital melanocytic nevus of the scalp despite clinical involution. J Am Acad Dermatol 2008;59:1091–1092.