Hox-C9 activates the intrinsic pathway of apoptosis and is associated with spontaneous regression in neuroblastoma

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Neuroblastoma is an embryonal malignancy of the sympathetic nervous system. Spontaneous regression and differentiation of neuroblastoma is observed in a subset of patients, and has been suggested to represent delayed activation of physiologic molecular programs of fetal neuralasts. Homeobox genes constitute an important family of transcription factors, which play a fundamental role in morphogenesis and cell differentiation during embryogenesis. In this study, we demonstrate that expression of the majority of the human HOX class I homeobox genes is significantly associated with clinical covariates in neuroblastoma using microarray expression data of 649 primary tumors. Moreover, a HOX gene expression-based classifier predicted neuroblastoma patient outcome independently of age, stage and MYCN amplification status. Among all HOX genes, HOXC9 expression was most prominently associated with favorable prognostic markers. Most notably, elevated HOXC9 expression was significantly associated with spontaneous regression in infant neuroblastoma. Re-expression of HOXC9 in three neuroblastoma cell lines led to a significant reduction in cell viability, and abrogated tumor growth almost completely in neuroblastoma xenografts. Neuroblastoma growth arrest was related to the induction of programmed cell death, as indicated by an increase in the sub-G1 fraction and translocation of phosphatidylserine to the outer membrane. Programmed cell death was associated with the release of cytochrome c from the mitochondria into the cytosol and activation of the intrinsic cascade of caspases, indicating that HOXC9 re-expression triggers the intrinsic apoptotic pathway. Collectively, our results show a strong prognostic impact of HOX gene expression in neuroblastoma, and may point towards a role of Hox-C9 in neuroblastoma spontaneous regression.

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Class I homeobox (Hox) transcription factors constitute an important family of developmental regulators, which play a fundamental role in morphogenesis and cell differentiation during embryogenesis.¹ In neural progenitors, HOX gene expression is controlled by retinoic acid (RA), fibroblast growth factors and wingless-type family members.²³ A deregulated expression of HOX genes has been observed in a number of malignancies.⁴–⁷

Neuroblastoma, an embryonal tumor of the sympathetic nervous system, originates from primordial neural crest cells, which are destined for sympathetic differentiation. This pediatric solid tumor shows remarkable variations in clinical presentations ranging from aggressive, therapy-resistant progression to spontaneous regression, which regularly occurs in infants both with localized and metastasized disease. Furthermore, neuroblastoma cells show the potential to differentiate toward a sympathetic ganglion cell phenotype.⁸ It has thus been suggested that the physiologic molecular program of neuroblast differentiation and growth control is disrupted in neuroblastoma.⁹ This developmental arrest may be reversible in spontaneously regressing neuroblastoma, in which a delayed activation of naturally occurring processes of programmed cell death has been suspected.¹⁰–¹² According to its embryonic nature, many genes aberrantly expressed in neuroblastoma are involved in developmental processes.¹³,¹⁴ Among others, several transcription factors involved in the development of autonomic neural crest derivatives, such as MYCN and PHOX2B, are implicated in the pathogenesis of neuroblastoma.¹⁵–¹⁸ Furthermore, various HOX genes have been described to be aberrantly

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Abbreviations: ABD, abdomen; Aber, aberration; aCGH, array-comparative genomic hybridization; AG, adrenal glands; amp, amplification; C, chest; Dox, doxycycline; DSMZ, German Collection of Microorganisms and Cell Cultures; EFS, event-free survival; fav, favorable; GO, gene ontology; GOMT, Gene Ontology Tree Machine; Hox, homeobox; IGV, integrative genomics viewer; Loc, localization; NK, neck; norm, normal; OS, overall survival; PAM, prediction analysis for microarrays; RA, retinoic acid; SR, spontaneously regressive; SVM, support vector machine; unfav, unfavorable

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expressed in neuroblastoma cell lines and primary tumors.\textsuperscript{19,20} In a recent study, Mao \textit{et al.}\textsuperscript{21} reported that \textit{HOXC9} expression is decreased in advanced-stage neuroblastoma and is involved in cell cycle control and the processes of neuroblasta
toma cell differentiation.

In this study, we aimed at determining the association of class I \textit{HOX} gene expression patterns with prognostic markers and outcome in neuroblastoma. Because \textit{HOXC9} was not only associated with favorable outcome but also with spontaneous regression, we investigated the functional consequences of \textit{HOXC9} re-expression on neuroblastoma growth and programmed cell death.

Results

Expression of the \textit{HOX} gene cluster is deregulated in neuroblastoma. The expression patterns of the 39 class I \textit{HOX} genes were analyzed in 649 neuroblastoma samples by microarrays, and the association with prognostic markers and patient outcome was determined. The expression of the majority of \textit{HOX} genes correlated significantly with clinical covariates in neuroblastoma. Elevated expression of \textit{HOXD} genes, particularly \textit{HOXD3}, \textit{HOXD8}, \textit{HOXD9} and \textit{HOXD10}, was predominantly associated with unfavorable prognostic markers and poor outcome (Supplementary Table S1 and Supplementary Figures S1 and S2). Similarly, we observed increased expression levels of more posterior \textit{HOX} genes (\textit{HOXA10}, \textit{HOXA11}, \textit{HOXA13}, \textit{HOXC12} and \textit{HOXC13}) in neuroblastomas with unfavorable characteristics (Supplementary Table S1). In contrast, elevated expression of the majority of the remaining \textit{HOX} genes was significantly correlated with favorable prognostic markers (Supplementary Table S1).

Prediction of neuroblastoma outcome based on a \textit{HOX} gene expression signature. To assess the impact of \textit{HOX} gene expression on neuroblastoma outcome, we developed a \textit{HOX} gene expression-based classifier (Table 1a and Supplementary Table S2) using a training cohort of 75 neuroblastoma patients with maximal divergent outcome. In the training set, the classification accuracy was 85\% as assessed by cross-validation. In a validation subset of 215 patients who matched the outcome criteria of the training set, the 33 \textit{HOX} gene signature predicted patient outcome with an accuracy of 76\% (Table 1b). In the entire validation set (\(n = 574\)), the classifier accurately discriminated patients with favorable and unfavorable outcome (favorable: \(n = 265\); 5-year event-free survival (EFS), 80.7\% \pm 2.6\%; 5-year overall survival (OS), 96.0\% \pm 1.2\%; unfavorable: \(n = 309\); 5-year EFS, 49.5\% \pm 3.0\%; 5-year OS, 67.1\% \pm 2.8\%; both \(P < 0.001\)). In multivariate Cox regression models based on EFS and OS, the \textit{HOX} classifier predicted patient outcome independently of age, stage and \textit{MYCN} amplification status (Table 1c).

Elevated \textit{HOXC9} expression is associated with favorable prognostic markers, beneficial patient outcome and spontaneous regression. Elevated \textit{HOXC9} expression levels strongly correlated with lower stages, age < 18 months at diagnosis, lack of \textit{MYCN} amplification, lack of 1p loss and favorable gene expression-based classification\textsuperscript{22,23} (Figure 1a). Kaplan–Meier estimates were calculated by

| Classifier | Patients (\(N\)) | Probes (\(N\)) | Accuracy (%) | Sensitivity (%) | Specificity (%) |
|------------|------------------|----------------|--------------|----------------|----------------|
| (a) | | | | | |
| NB_d75_hox52_fsPAM_pred | 75 | 52 | 85 | 58 | 96 |
| (b) | | | | | |
| NB_d75_hox52_fsPAM_pred | 215 | 40 | 76 | 63 | 90 |

| Marker | Patients (\(N\)) | Available cases (\(N\)) | Hazard ratio | 95\% CI | \(P\)-value |
|--------|------------------|--------------------------|--------------|---------|-------------|
| (c) | | | | | |
| Model considering single prognostic markers and the \textit{HOX} classifier based on EFS | 574 | 547 | | | |
| Age (< 18 months versus > 18 months) | 2.06 | 1.48–2.87 | <0.001 |
| Stages (1–3, 4S versus 4) | 1.49 | 1.49–2.07 | 0.018 |
| \textit{MYCN} (amplified versus normal) | 1.44 | 1.44–2.03 | 0.041 |
| \textit{HOX} classifier (favorable versus unfavorable) | 2.13 | 1.48–3.06 | <0.001 |

| Model considering single prognostic markers and the \textit{HOX} classifier based on OS | 574 | 568 | | | |
| Age (< 18 months versus > 18 months) | 4.44 | 2.61–7.53 | <0.001 |
| Stages (1–3, 4S versus 4) | 2.24 | 1.39–3.58 | <0.001 |
| \textit{MYCN} (amplified versus normal) | 2.62 | 1.77–3.88 | <0.001 |
| \textit{HOX} classifier (favorable versus unfavorable) | 3.1 | 1.67–5.76 | <0.001 |

Abbreviation: CI, confidence interval.
using two independent neuroblastoma patient cohorts. The first cohort consisted of 244 neuroblastoma patients, which has been published previously by our group\textsuperscript{22} and has been used by Mao and co-workers\textsuperscript{21} for clinical analysis of HOXC9 expression. This cohort was used to determine cutoff values of HOXC9 expression levels by quartiles. The cutoff values were applied on an independent set of 405 neuroblastoma patients to determine clinical courses of patients with high, intermediate–high, intermediate–low and low HOXC9 expressions. Patients with high HOXC9 expression had a significantly better outcome with a 5-year OS of 95 ± 2% as compared to patients with low HOXC9 expression (5-year OS, 56 ± 5%; Figure 1b). Likewise, HOXC9 expression levels strongly correlated with improved EFS (high HOXC9 expression, 5-year EFS, 82 ± 4% versus low HOXC9 expression, 5-year EFS, 37 ± 5%; Figure 1c). In addition, multivariate Cox regression models based on EFS and OS, considering established risk markers (MYCN status, tumor stage and patient age at diagnosis), determined HOXC9 expression as a significant independent prognostic marker for both EFS and OS (Table 2). We also investigated HOXC9 expression levels in infant neuroblastoma that had regressed without any chemotherapy, and compared these with infant stage 4 neuroblastoma. Notably, HOXC9 transcript levels were significantly higher in neuroblastoma showing spontaneous regression (Figure 1d and Supplementary Table S3). Taken together, these results demonstrate that HOXC9 transcript levels discriminate neuroblastoma patients with favorable and unfavorable outcome and indicate that elevated HOXC9 expression is associated with spontaneous regression.

Elevated HOXC9 expression is associated with abdominal neuroblastoma. As the expression of HOX genes is not only temporally but also spatially regulated during embryonic development, HOXC9 expression were compared in neuroblastoma according to the primary tumor site. We observed significantly higher HOXC9 expression levels in tumors with abdominal location in comparison to neuroblastomas located at the neck/chest or at adrenal glands (Figure 1e), which is in line with the embryonal expression pattern of HOX genes: HOX genes located towards the 5' end of the HOX cluster, such as HOXC9,
are expressed more posterior in the body during development. Analogous expression patterns, however, were observed for only few of the remaining 5’-located HOX genes (Supplementary Figure S3a). Interestingly, the spatial expression pattern of HOXC9 might not have been expected from the association of primary tumor sites with patient outcome.24,25 In line with these reports, we observed a better outcome of patients with neck/chest neuroblastoma in comparison to tumors located at the adrenal glands or in the abdomen (neck/chest versus adrenal glands versus abdomen, 5-year EFS, 84 ± 5% versus 69 ± 3% versus 63 ± 4%, respectively; 5-year OS, 97 ± 2% versus 83 ± 2% versus 85 ± 3%, respectively; Supplementary Figures S3b and S3c).

**Numerical gain of chromosome 12 correlates with elevated expression of HOXC9.** We next aimed to evaluate whether genetic or epigenetic aberrations of the HOXC9 locus are associated with deregulated HOXC9 expression. First, we analyzed array-comparative genomic hybridization (aCGH) profiles of 209 neuroblastoma samples and compared the results with corresponding microarray gene expression data of the same tumors. Genomic aberrations of chromosome 12 were detected in 92/209 (44.0%) of the samples (Figure 2a and Supplementary Table S4). We most frequently observed numerical gains of the entire chromosome (n = 64, 30.6%). Numerical chromosome 12 loss occurred only in four cases. Segmental alterations of chromosome 12 were detected in 24 tumors (11.5%), most of which were segmental gains not affecting the HOXC9 locus (n = 18, 8.6%). Analysis of HOXC9 gene expression data revealed significantly higher transcript levels in tumors with numerical chromosome 12 gains in comparison to tumors in which the HOXC9 locus was not affected by genomic alterations (Figure 2b).

**Table 2** Multivariate Cox regression models based on EFS and OS considering age at diagnosis (> 18 months versus ≤ 18 months), stage (4 versus 1–3, 4S), MYCN status (amplified versus normal) and HOXC9 expression (continuous)

| Marker                                                                 | Patients (N) | Available cases (N) | Hazard ratio | 95% CI      | P-value |
|------------------------------------------------------------------------|--------------|---------------------|--------------|-------------|---------|
| Model considering single prognostic markers and HOXC9 expression based on EFS | 649          | 622                 | 0.48         | 0.34–0.66   | <0.001  |
| Age (> 18 months versus ≤ 18 months)                                   |              |                     | 0.52         | 0.38–0.72   | <0.001  |
| MYCN (amplified versus normal)                                         |              |                     | 0.57         | 0.42–0.79   | 0.001   |
| HOXC9 expression (continuous)                                          |              |                     | 0.75         | 0.60–0.93   | 0.012   |
| Model considering single prognostic markers and HOXC9 expression based on OS | 649          | 643                 | 0.24         | 0.15–0.39   | <0.001  |
| Age (> 18 months versus ≤ 18 months)                                   |              |                     | 0.33         | 0.26–0.52   | <0.001  |
| MYCN (amplified versus normal)                                         |              |                     | 0.32         | 0.22–0.46   | <0.001  |
| HOXC9 expression (continuous)                                          |              |                     | 0.73         | 0.55–0.97   | 0.03    |

Abbreviations: CI, confidence interval

**Figure 2** Aberrations at chromosome 12 and correlation with HOXC9 expression. (a) Chromosomal aberrations of the HOXC9 locus in primary neuroblastomas (n = 200). (b) Association of whole chromosome 12 gain with HOXC9 expression in primary neuroblastomas. Aberr, aberration; NG, numerical gain
To examine whether inactivating mutations might contribute to diminished \( \text{HOXC9} \) expression in neuroblastoma, we sequenced the genomic \( \text{HOXC9} \) locus of 46 primary tumors with low \( \text{HOXC9} \) expression. A total of 16 unique sequence variants were detected (Supplementary Table S5). Eight of these represented known SNPs, whereas the remaining sequence variants were novel. The latter variants affected either non-coding sequences or were synonymous, and occurred infrequently in our cohort. Taken together, these data suggest that mutations in the \( \text{HOXC9} \) locus are not a major cause of reduced \( \text{HOXC9} \) expression levels in unfavorable neuroblastoma.

To determine whether epigenetic regulation might contribute to differences in \( \text{HOXC9} \) expression in neuroblastoma, we analyzed the methylation status of 26 CpG sites located in the \( \text{HOXC9} \) promoter region. DNA samples from 46 neuroblastoma tumors with differing \( \text{HOXC9} \) expression levels (high and low \( \text{HOXC9} \) expression as defined in Materials and Methods, \( n = 23 \) per subgroup) and 2 neuroblastoma cell lines (SK-N-AS and IMR-32) with loss of \( \text{HOXC9} \) expression were analyzed. Unsupervised one-way hierarchical clustering of CpG site methylation revealed a largely homogeneous methylation pattern in tumors of both subgroups and cell lines (Supplementary Figure S4b), suggesting that down-regulation of \( \text{HOXC9} \) does not result from altered CpG methylation patterns.

**Doxycycline-inducible expression of Hox-C9 in neuroblastoma cell lines.** \( \text{HOXC9} \) expression was restored in three neuroblastoma cell lines. Polyclonal Hox-C9-expressing neuroblastoma cells were compared with polyclonal GFP-expressing cells (Figure 3a) due to promoter leakage of the pRevTRE Vector System (Figure 3b). Recombinant Hox-C9 protein levels were comparable to physiological protein levels observed in neuroblastoma patients with high Hox-C9 expression (Figure 3c).

**HOXC9 expression inhibits growth of neuroblastoma cells in vitro and in vivo.** To investigate whether \( \text{HOXC9} \) expression affects growth properties in neuroblastoma cells, polyclonal \( \text{HOXC9} \)-expressing IMR-32, SK-N-AS and CHP-212 cells were compared with polyclonal GFP-expressing controls. Cell proliferation was assayed for up to 8 days using Trypan blue dye exclusion tests. In all cell lines, the number of viable cells at day 8 was significantly lower compared with GFP-induced controls (Figure 4a). To determine whether reduced proliferation upon \( \text{HOXC9} \) re-expression might be due to impaired cell cycle progression, the DNA content of \( \text{HOXC9} \)-expressing cells was assessed by flow cytometry. We observed a significant increase of the G0/G1 peak at day 7 after Hox-C9 induction in all three cell lines (Figure 4b). To investigate the influence of \( \text{HOXC9} \) on anchorage-independent clonal growth, we performed soft agar assays. A marked reduction in colony formation was observed in both SK-N-AS and IMR-32 cells in comparison to control cells (Figure 4c).

**Hox-C9-induced neuronal differentiation of IMR-32 cells is accompanied by the downregulation of REST.** Morphological signs of neuronal differentiation were assessed by microscopic examination in all three cell lines after \( \text{HOXC9} \) re-expression. In IMR-32 cells, \( \text{HOXC9} \) re-expression led to a neuronal-like phenotype with a large network of cells interconnected by long neurite elongations (Figure 5a), while similar changes were not observed in the...
other cell lines. To determine whether this morphological alteration induced by Hox-C9 was accompanied by the upregulation of genes involved in neuronal differentiation, we analyzed expression levels of the neuron-related markers DNER, NTS, PTN, NNAT, TRKA and NEFL by quantitative real-time reverse transcriptase-polymerase chain reactions (qRT-PCR) and microarray analysis. In line with the morphological changes, all markers were upregulated upon HOXC9 re-expression (Figures 5b and c). A similar trend was observed for additional markers associated with neuronal differentiation (Supplementary Figure S5a and S5b). At the same time, the RE1-silencing transcription factor REST, a master negative regular of neurogenesis, was downregulated in Hox-C9-induced IMR-32 cells (Figures 5d and e).

**HOXC9 re-expression induces apoptosis in neuroblastoma cells.** We next examined whether apoptosis contributes to the inhibition of neuroblastoma cell growth after HOXC9 re-expression. First, externalization of phosphatidylserine was analyzed by flow cytometry using Annexin-V staining. We observed a significant increase of the Annexin-V-binding fraction in SK-N-AS and CHP-212 cells 168 h after HOXC9 re-expression in comparison to control cells (Figure 6a). Second, DNA fragmentation was assessed using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay in SK-N-AS, IMR-32 and CHP-212 cells. HOXC9-expressing neuroblastoma cells showed a significantly higher fraction of apoptotic cells as compared with GFP-expressing controls (Figure 6b). These results were supported by FACS analysis following Hox-C9 induction in SK-N-AS, IMR-32 and CHP-212 cells, in which Nicoletti labeling and assessment of the fraction of sub-G1 events indicated an accumulation of cells with fragmented DNA. In all investigated cell lines, the number of apoptotic cells was significantly increased by HOXC9 re-expression as compared with GFP-induced controls (Figure 6c). Taken together, these findings indicate that Hox-C9 affects growth properties in human neuroblastoma cells not only by cell cycle regulation but also by induction of apoptosis.

**HOXC9 activates the intrinsic pathway of apoptosis.** To further analyze Hox-C9-induced cell death, the expression of pro- and antiapoptotic regulators was examined by immunoblot, qRT-PCR and microarray analysis. Apoptotic cell death was associated with a strong increase of cytochrome c in the cytosolic fraction of Hox-C9-induced cells (Figure 6d), suggesting a loss of mitochondrial membrane potential and involvement of mitochondria in Hox-C9-induced cell death. In IMR-32 cells, we observed downregulation of BCL-2 at both the transcript and the protein level, while the expression levels remained unchanged in SK-N-AS and CHP-212 cells (Figures 6d–f). In the latter cell lines, however, we observed a marked upregulation of BAX mRNA expression upon induction of Hox-C9 (Figures 6e and f). These data suggest that apoptosis induced by HOXC9 re-expression may be conferred by a shifted BCL-2/BAX ratio as a consequence of either increased expression of proapoptotic BAX or decreased expression of antiapoptotic Bcl-2. Subsequently, we investigated whether procaspase-9, the initial caspase in the mitochondrial apoptotic cascade, is activated upon
cytochrome c release. We observed an increase of cleaved 35 kDa fragments of activated caspase-9 in all cell lines upon HOXC9 re-expression (Figure 6d). In addition, a 37 kDa cleaved fragment of caspase-9 was observed in SK-N-AS and CHP-212 cells (Figure 6d), which is indicative of a feedback amplification loop induced by activated caspase-3. Finally, we observed an increased activation of the downstream effector caspase-3 and -7 in all three cell lines (Figure 6d) upon HOXC9 re-expression. Taken together, these data indicate that HOXC9 expression can activate the intrinsic pathway of apoptosis and thereby trigger neuroblastoma cell death.

Hox-C9 affects transcriptional pathways regulating differentiation and cell death. To gain further insights into the molecular processes occurring upon HOXC9 re-expression, we analyzed gene expression profiles of IMR-32 and SK-N-AS cells after Hox-C9 induction using microarrays. We used gene ontology (GO) annotations to find classes of genes that are significantly over-represented in gene sets that were either up- or downregulated after HOXC9 re-expression (Supplementary Table S6). Many GO categories significantly enriched for genes upregulated in HOXC9-expressing IMR-32 cells were related to neuronal functions and differentiation, thereby reflecting the Hox-C9-associated differentiation phenotype in IMR-32 cells (Supplementary Table S6 and Supplementary Figure S7a). In SK-N-AS cells, we observed GO categories significantly enriched for genes upregulated by Hox-C9 that were associated with cell death (Supplementary Table S6 and Supplementary Figure S7c). These results are well in line with the observed phenotypes of these two neuroblastoma cell lines after HOXC9 re-expression: While growth inhibition mediated by apoptosis is the predominant characteristic of
HOXC9-expressing SK-N-AS cells, neuronal differentiation is mainly observed in IMR-32 cells.

Discussion
In this study, we show that expression of the majority of class I HOX genes is associated with clinical phenotypes of neuroblastoma. HOX genes play essential roles in morphogenesis and cell differentiation during embryonic development. As neuroblastoma is an embryonal tumor capable of differentiating into ganglioneuroma or ganglioneuroblastoma, it has been suggested that HOX transcription factors may be involved in these processes. Accordingly, it has been reported that morphological differentiation of neuroblastoma cells upon treatment with RA is accompanied by an increase of expression of a number of HOX genes. In line with these results, we show here that the expression of most HOX genes is significantly associated with prognostic markers and clinical outcome in primary neuroblastoma, suggesting that HOX genes are essentially involved in neuroblastoma pathogenesis. The potential relevance of these genes is further emphasized by the finding that a HOX gene expression-based classifier was able to predict accurately neuroblastoma patient outcome independently of age, stage and

Figure 6  Hox-C9 induces apoptosis in neuroblastoma cell lines by activating the intrinsic apoptotic pathway. (a) Proportion of Annexin-V-positive cells as determined by fluorescence-activated cell sorting (FACS). (b) Proportion of cells showing apoptotic nuclei as determined by fluorescence microscopy. (c) Proportion of cells with subnormal DNA content as determined by FACS. (d) Protein expression of Bcl-2, cytosolic cytochrome c and cleaved caspase-9, caspase-7 and caspase-3 upon Hox-C9 and GFP induction as determined by western blot analysis (for densitometric quantification see Supplementary Figure S6). (e) BCL-2 and BAX expression in IMR-32 and SK-N-AS cells following Hox-C9 induction as determined by microarrays. (f) BCL-2 expression in IMR-32 cells, and BAX expression in SK-N-AS and CHP-212 cells following Hox-C9 induction as determined by qRT-PCR. Error bars indicate S.D. Dox, doxycycline
MYCN status. We observed that HOX genes of the A, B and C clusters were mainly upregulated in favorable neuroblastoma, while genes of the D cluster, particularly HOXD3, HOXD8, HOXD9 and HOXD10, were upregulated in unfavorable tumors. These findings are in contrast to a recent study of Mao et al., who investigated the prognostic values of a panel of HOX genes in primary neuroblastoma, and did not observe any correlation of expression and clinical outcome for most of these. The difference between the two studies might be explained by the fact that Mao and co-workers had examined a limited number of samples comprising metastasis-sized neuroblastoma without MYCN amplification only, while a large tumor cohort representing the entire spectrum of the disease has been analyzed in our study.

Among all HOX genes, HOXC9 expression was most prominently associated with favorable prognostic markers and with clinical outcome. The prognostic impact of HOXC9 is underlined by the fact that this gene was selected in all predictive models generated during the training phase of the process of HOX classifier generation, and that it was one of 144 genes of a prognostic classifier that we have reported previously. Using gene expression data from our group and others, HOXC9 expression has in addition been described to be associated with the clinical neuroblastoma phenotype in a recent study. Notably, we observed that HOXC9 expression levels were higher in neuroblastomas, which had spontaneous regressed than in age-related stage 4 tumors, suggesting that this transcription factor might be involved in the enigmatic molecular process of spontaneous regression.

Spatial and temporal collinearity is a hallmark of clustered HOX genes. HOX genes that are positioned closer to the 5′ end of the cluster, such as HOXC9, are expressed later and more posterior during early development. In accordance with the embryonic expression pattern of HOX genes, we here show a significant correlation of increased HOXC9 expression levels of neuroblastomas from abdominal sites in comparison to tumors of the neck and chest. This finding contrasts with previous speculations, which suggested that this transcription factor might be involved in the enigmatic molecular process of spontaneous regression.

HOXC9 expression in neuroblastoma. A gain of chromosome 12, which comprises the HOXC9 locus, was associated with elevated HOXC9 transcript levels, suggesting that a gene–dosage effect might account for HOXC9 expression differences in neuroblastoma. These findings are in line with previous reports showing that numerical chromosomal aberrations, including chromosome 12 gain, are associated with lower stages and better outcome in neuroblastoma. By contrast, we neither detected hypermethylation of the promoter region of HOXC9 nor inactivating mutations in the HOXC9 locus as a potential cause of diminished HOXC9 expression in unfavorable tumors. Alternatively, other mechanisms, such as regulatory RNAs or upstream transcriptional regulators, may control HOXC9 expression in neuroblastoma.

In vivo and in vitro expression in neuroblastoma. In line with data reported by Mao et al., we observed reduced proliferation and cell cycle arrest in the G0/G1 phase upon HOXC9 re-expression. Moreover, we substantiate that HOXC9 may induce neuronal differentiation in a fraction of neuroblastomas. In addition, we observed downregulation of REST after HOXC9 re-expression. REST is a well-known master negative regulator of neurogenesis and has been reported to be degraded upon RA-induced neuroblastoma cell differentiation. Taking these findings together, it seems likely that Hox-C9 promotes a more differentiated neoblastoma phenotype by the downregulation of REST.

The strong effect of HOX-C9 on neuroblastoma growth arrest observed by us and others suggested that other mechanisms than cell cycle arrest alone may contribute to this phenotype. Accordingly, we here provide substantial evidence that Hox-C9 promotes apoptosis in neuroblastoma in general, as indicated by a strong increase in the sub-G1 fraction and an increase in TUNEL-positive cells after HOX-C9 induction in all three cell lines. We demonstrate that HOXC9 expression activates the intrinsic pathway of apoptosis, probably by increasing the BAX:BCL-2 ratio, which in turn leads to the release of cytochrome c from the mitochondria into the cytosol and activates the intrinsic cascade of caspases. Delayed activation of the naturally occurring process of programmed cell death during development of the peripheral nervous system has been suggested to represent the molecular basis of spontaneous regression in neuroblastoma. Taking the correlation of HOXC9 expression with favorable outcome and spontaneous regression in neuroblastoma, its developmental significance and its proapoptotic function in neuroblastoma cell lines into account, we hypothesize that Hox-C9 may contribute to the process of spontaneous regression in neuroblastoma.

In summary, we here demonstrate that distinct HOX gene expression patterns are associated with clinical phenotypes of neuroblastoma. We show that HOXC9 expression does not only correlate with favorable prognostic markers and beneficial clinical outcome but also with spontaneous regression in infant neuroblastoma. Re-expression of HOXC9 in neuroblastoma cell lines consistently leads to strong growth arrest and activates the intrinsic pathway of apoptosis. Taken together, we conclude from our data that HOX genes may contribute to neuroblastoma pathogenesis, and that Hox-C9 might be involved in the molecular process of spontaneous regression.

**Materials and Methods**

**Patient characteristics.** Tumors of 649 neuroblastoma patients were analyzed. Stages were classified according to the International Neuroblastoma Staging System. Primary tumors were localized in the abdomen (n = 135), at the neck/chest (n = 69) or the adrenal glands (n = 276). The primary tumor localization for the remaining 169 patients was unknown. The subgroup of patients showing regression of neuroblastoma consisted of 43 infants (localized, n = 14; stage 4S, n = 78). Primary tumors were localized in the abdomen (n = 135), at the neck/chest (n = 69) or the adrenal glands (n = 276). The primary tumor localization for the remaining 169 patients was unknown. The subgroup of patients showing regression of neuroblastoma consisted of 43 infants (localized, n = 14; stage 4S, n = 78). In the tumor manifestations showed unambiguous regression without any cytotoxic treatment. The neuroblastoma subgroups used for methylation and aCGH analyses consisted of 46 and 209 tumors, respectively. Risk estimation of corresponding patients was performed according to the International Neuroblastoma Risk Group (INRG) classification system (methylation: high risk, n = 21; intermediate and low risk, n = 24; unclassified, n = 1; aCGH: high risk, n = 56; intermediate and low risk, n = 153).

**Gene expression analysis.** Single-color gene expression profiles from 649 neuroblastoma tumors and 2 neuroblastoma cell lines were generated using 44K
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Neuroblastoma cell lines were authenticated at the DSMZ. Cells were maintained as described in Supplementary Materials and Methods.

Retroviral plasmids and stable inducible neuroblastoma cell lines. A human HoxC9 full open reading frame was obtained from the retroviral vector pBlGzHoxC9 (kind gift of Malte Buchholz, Philips-University of Marburg, Marburg, Germany) and cloned into the pRevTRE vector (Clontech, Heidelberg, Germany; Supplementary Materials and Methods). Polyclonal neuroblastoma cell lines stably expressing either HoxC9 or GFP under the control of the reverse tetracycline-controlled transactivator were generated using the RevTet System (Clontech) according to the manufacturer’s instructions with marginal modifications (Supplementary Materials and Methods).

Expression analyses using immunoblots and qRT-PCR. Immunoblots were prepared as described previously.53 Cytosolic cell extracts were isolated as described elsewhere.50 Total protein extracts were isolated either with RIPA or Cell Lysis Buffer (Biovision, Hannover, Germany). Densitometric quantification of immunoblots was performed using the Image J software (NIH, Bethesda, MD, USA).53 qRT-PCR was carried out twice in duplicates as described previously.52 Antibody and primer information is given in Supplementary Materials and Methods and Supplementary Table S5, respectively.

In vitro growth assays. Cell viability was analyzed by Trypan blue dye exclusion (Supplementary Materials and Methods) and cell cycle distribution was assessed by FACS analysis as described previously.53

Tumorigenicity assays. Soft agar assays were used to assess the effect of HoxC9 on colony formation ability of neuroblastoma cells (Supplementary Materials and Methods). A total of 32 six-week-old female athymic nude-Foxn1nu mice (Harlan Laboratories, Horst, The Netherlands) were used for the establishment of neuroblastoma xenograft tumors (Supplementary Materials and Methods).

Cell viability analysis. The TUNEL analysis was performed using the In Situ Cell Death Detection Kit according to the manufacturer’s protocol (Roche, Mannheim, Germany; Supplementary Materials and Methods). Annexin-V-binding analysis was carried out using APC-coupled Annexin-V and 7-AAD according to the manufacturer’s protocol (BD Biosciences, Heidelberg, Germany). Apoptotic cells were detected by FACS (FACS Canto; BD Biosciences) using the DIVA software (BD Biosciences). The extent of DNA fragmentation was evaluated in a hypoploidy assay as described elsewhere.52

Conflict of Interest

The authors declare no conflict of interest.

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1. Gehring WJ, Hiromi Y. Homeotic genes and the homeobox. Annu Rev Genet 1986; 20: 147–173.
2. Bel-Vialar S, Itasaki N, Kruimla R. Initiating Hox gene expression: in the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups. Development 2002; 129: 5103–5115.
3. Nordstrom U, Maier E, Jessell TM, Edlund T. An early role for WNT signaling in specifying neural patterns of Cdx and Hox gene expression and motor neuron subtype identity. PLoS Biol 2006; 4: e292.
4. Daniels TR, Necatco II, Rodriguez JA, Pandha HS, Morgan R, Penichet ML. Disruption of Hox activity leads to cell death that can be enhanced by the interference of iron uptake in malignant B cells. Leukemia 2010; 24: 1555–1565.
5. Shears I, Plowright L, Harrington K, Pandha HS, Morgan R. Disrupting the interaction between HOX and PBX causes necrotic and apoptotic cell death in the renal cancer lines CaK-2 and 769-P. J Urol 2008; 180: 2196–2201.

Oligonucleotide microarrays as described previously.43 Total RNA of HoxC9- and GFP-expressing SK-N-AS and IMR-32 cells was isolated at 0, 6, 12, 24, 48 and 96 h using Trizol (Invitrogen, Karlsruhe, Germany). To determine global differences in the expression profiles of HoxC9- and GFP-induced SK-N-AS and IMR-32 cells, mean expression levels of each gene between HoxC9-induced and control cells were compared. Gene Ontology Tree Machine (GOTM)44 was used to identify functional categories associated with the condition of the respective cell line (Supplementary Materials and Methods). All raw and normalized microarray data are available through the Gene Expression Omnibus database (Accession: GSE45480).

Hox gene expression-based classification. To develop a Hox gene expression-based classifier (Hox classifier), the nearest shrunken centroids method (PAM)45 was applied on Hox gene expression data from 75 neuroblastoma patients with maximal divergent clinical outcome.46 Classification performance in the training set was evaluated by a 10 times repeated 5-fold cross-validation. Probes that were included in at least 65% of all cross-validation models were selected as classifier probes.47 Classification accuracy of the final Hox classifier comprising 33 Hox genes was assessed in an independent validation set of 574 neuroblastoma patients using the support vector machine (SVM)46 algorithm. Multivariate Cox proportional hazard models based on EFS and OS were used to analyze the prognostic impact of this classifier. The factors age (<18 months versus >18 months), tumor stage (stages 1–3 versus 4 versus stage 4), MYCN (normal versus amplified) and Hox classifier were fitted into a stepwise-forward selection model.

Analysis of genetic aberrations and promoter methylation of HoxC9. Oligonucleotide aCGH profiles from 209 neuroblastoma tumors were generated using 44K, 105K or 1M microarrays as described previously.35,47 Array-CGH analysis was performed as described previously48 and visualized using the Integrative Genomics Viewer.49 Because of technical reasons, 9 out of 209 aCGH profiles could not be visualized. Array-CGH data are available at Gene Expression Omnibus (Accession: GSE25771 and GSE45480). HoxC9 promoter methylation analysis was performed by Sequenom Inc. (Hamburg, Germany; Supplementary Materials and Methods and Supplementary Table S5). Whole genome-amplified DNA from primary neuroblastoma specimens and neuroblastoma cell lines were used to sequence amplicons covering the first 500 nucleotides upstream of the HoxC9 start site, 5’- and 3’-UTRs and the complete coding region with exon–intron boundaries. Sequencing was performed by Beckman Coulter Genomics (Danvers, MA; USA; Supplementary Materials and Methods and Supplementary Table S5).

Data analysis and statistics. Statistical analysis of associations between Hox mRNA expression levels and neuroblastoma subgroups, prognostic markers and outcome in neuroblastoma was performed using SPSS version 20.0 (IBM, Mainz, Germany). Two-tailed non-parametric tests (Mann–Whitney U-test and Kruskal–Wallis test) were used when appropriate. All Hox genes were first tested in a univariable Cox regression model based on OS and EFS. For all Hox genes with $P$ values $\leq 0.05$, the whole cohort (n = 649) was divided using quartiles based on Hox gene expression levels. Kaplan–Meier estimates for OS (n = 649) and EFS (n = 628) were compared by log-rank test. In addition, HoxC9 was investigated separately by using quartile values of HoxC9 expression levels in a set of 244 neuroblastoma tumors. These values were applied on an independent set of 405 neuroblastoma expression profiles as a cutoff for high, intermediate–high, intermediate–low and low HoxC9 expressions. Kaplan–Meier estimates for OS (n = 405) and EFS (n = 384) were compared by log-rank test. Recurrence, progression and death from disease were considered as events. Multivariate Cox proportional hazard models based on EFS and OS were used to analyze the prognostic impact of HoxC9 expression in neuroblastoma. The factors age (>18 months versus <18 months), tumor stage (stage 4 versus stages 1–3 and 4S), MYCN (amplified versus normal) and HoxC9 expression were fitted into a stepwise-backward selection model. The likelihood ratio test $P$ for inclusion was $\leq 0.05$ and for exclusion was $>0.05$. Quantitative data for functional analysis were shown as means ± S.D. Unpaired two-tailed Student’s t-tests were used where appropriate.

Cell culture. SK-N-AS and CHP-212 cells were obtained from ATCC (Manassas, VA, USA) and IMR-32 cells were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).
6. Plowright L, Harrington KJ, Pandha HS, Morgan R. HOX transcription factors are potential therapeutic targets in non-small-cell lung cancer (targeting HOX genes in lung cancer). Br J Cancer 2009; 100: 470–475.

7. Abate-Shen C. Deregulated homeobox gene expression in cancer: cause or consequence? Nat Rev Cancer 2002; 2: 777–786.

8. Shimada H, Ambros IM, Dehner LP, Hata J, Joshu VW, Roald B. Terminology and morphologic criteria of neuroblastoma: recommendations by the International Neuroblasteoma Pathology Committee. Cancer 1999; 86: 36–393.

9. Nakagawara A, Shimada H, Chatten J, Newton WA Jr., Sachs N, Hamoudi AB, Chiba T. Neural crest development and neuroblastoma: the genetic and biological significance of gene expression profiles of metastatic neuroblastoma. J Clin Oncol 2009; 27: 1292–1303.

10. van Noesel MM. Neuroblastoma stage 4S: a multifocal stem-cell disease of the developing neural crest. Lancet Oncol Mar 2012; 13: 229–230.

11. Pritchard J, Holman JA. Why does stage 4S neuroblastoma regress spontaneously? Lancet 2006; 367: 869–870.

12. Nakagawara A. Neural crest development and neuroblastoma: the genetic and biological link. Prog Brain Res 2004; 146: 233–242.

13. Nakagawara A, Ohira M. Comprehensive genomics linking between neural development and cancer: neuroblastoma as a model. Cancer Lett 2004; 204: 213–224.

14. Fischer M, Oberthuer A, Bros B, Kahyel Y, Skowron M, Voth H. Differential expression of neuronal genes defines subsets of disseminated neuroblastoma with favorable and unfavorable outcome. Clin Cancer Res 2006; 12: 5118–5128.

15. Mosse YP, Lautdansler M, Kazhi D, Carlisle AJ, Winter CL, Rappaport E et al. Germline PHOX2B mutation in hereditary neuroblastoma. Am J Hum Genet 2004; 75: 727–730.

16. Park T, Moen X, Cramer H, Gobert C, Brunet JF. The homeobox gene PHOX2B is essential for the development of autonomic neural crest derivatives. Nature 1999; 399: 366–370.

17. Schwab M, Allkut A, Klemmperaur KH, Varmus HE, Bishop JM, Gilbert F et al. Amplified DNA with limited homology to mcv cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 1983; 305: 245–248.

18. Seeger RC, Broeder GM, Sather H, Dalton A, Siegel SE, Wong KY et al. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 1985; 313: 1111–1116.

19. Alimimos M, Mora J, Cheung NK, Smith A, Qin J, Chen L et al. Genome-wide analysis of gene expression associated with MYCN in human neuroblastoma. Cancer Res 2003; 63: 4538–4546.

20. Manohar CF, Furtado MR, Salwen HR, Cohn SL. Hox gene expression in differentiating human neuroblastoma cells. Biochem Mol Biol Int 1993; 30: 733–741.

21. Mao L, Ding J, Zha Y, Yang L, McCarthy BA, King W et al. Differentiation of neuroblastoma preserves the genetic complexity of neuroblastomas. Cancer Res 2004; 61: 62–69.

22. Plowright L, Harrington KJ, Pandha HS, Morgan R. HOX transcription factors are potential therapeutic targets in non-small-cell lung cancer (targeting HOX genes in lung cancer). Br J Cancer 2009; 100: 470–475.

23. Oberthuer A, Hero B, Berthold F, Juraeva D, Faldum A, Kahlert Y. The master negative regulator REST/PHOX2B mutation in hereditary neuroblastoma. Cell Death and Disease 2011; 2: 5118–5128.

24. Singh A, Rokes C, Gereud M, Fletcher S, Baumgartner J, Fuller G et al. Retinoid acid induces REST degradation and neuronal differentiation by modulating the expression of SCF(beta-TRCP) in neuroblastoma cells. Cancer 2011; 117: 5189–5202.

25. Broeder GM, Pritchard J, Berthold F, Carlsen NL, Castel V, Castellon RP et al. Revisions of the international criteria for neuroblastoma: diagnosis, staging, and response to treatment. J Clin Oncol 1993; 11: 1466–1477.

26. Hero B, Simon T, Spitz R, Eustein K, Gneikow AK, Scheel-Walter HG et al. Localized infant neuroblastomas often show spontaneous regression: results of the prospective trials NB95-S and NB97. J Clin Oncol 2008; 6: 1504–1510.

27. Cohn SL, Pearson AD, London WB, Moudair TA, Ambros PF, Broeder GM et al. The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. J Clin Oncol 2006; 25: 289–297.

28. Oberthuer A, Juraeva D, Li L, Kahlert Y, Westermann F, Eils R et al. Integrated genomic profiling identifies two distinct molecular subtypes with divergent outcome in neuroblastoma patients. Pharmacogenomics J 2010; 10: 258–266.

29. Zhang B, Schmoyer D, Kirov S, Snoddy J. GOtree Machine (GOTM): a web-based platform for interpreting sets of interesting genes using Gene Ontology hierarchies. BMC Bioinform 2004; 5: 16.

30. Tshibruni R, Hastele T, Narasimhan B, Chu G. Diagnosis of multiple cancer types by shrinked centroids of gene expression. Proc Natl Acad Sci USA 2002; 99: 6677–6727.

31. Vapnik VN. An overview of statistical learning theory. IEEE Trans Neural Netw 1999; 10: 988–999.

32. Fischer M, Bauer T, Oberthuer A, Hero B, Theissen J. Ehrich M et al. Integrated genomic profiling identifies two distinct molecular subtypes with divergent outcome in neuroblastoma with loss of chromosome 11q. Oncogene 2008; 29: 865–875.

33. Robinson JT, Thovaiidiotto H, Winckler W, Guttman M, Lander ES, Getz G et al. Integrative genomic view. Nat Biotechnol 2011; 29: 24–26.

34. Akermann S, Goessler F, Schuitte JH, Schramm A, Ehemann V, Hero B et al. Poly-Iike kinase 1 is a therapeutic target in high-risk neuroblastoma. Clin Cancer Res 2011; 17: 731–741.

35. Khashkar H, Kornek M, Jurgensmeier JM. Defective Bax activation in Hotchk B-cell lines confers resistance to staurosporine-induced apoptosis. Cell Death Differ 2002; 9: 750–757.

36. Schneider CA, Rasband WS, Elston RW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012; 9: 671–675.

37. Fischer M, Skowron M, Berthold F. Reliable transcript quantification by real-time reverse transcriptase-polymerase chain reaction in primary neuroblastoma using normalization to averaged expression levels of the control genes HPRT1 and SDHA. J Mol Diagn 2005; 7: 89–98.

38. Ehemann V, Hashemi B, Lange A, Otto FH. Flow cytometric DNA analysis and chromosomal aberrations in malignant glomusomas. Cancer Lett 1999; 138: 101–106.

39. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991; 139: 271–279.

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