Homeobox genes in neuroblastoma
Revet, I.M.

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The MSX1 homeobox transcription factor is a downstream target of PHOX2B and activates the Delta-Notch pathway in neuroblastoma

Ingrid Revet, Gerda Huizenga, Alvin Chan, Jan Koster, Richard Volckmann, Peter van Sluis, Ingrid Øra, Rogier Versteeg, and Dirk Geerts

Department of Human Genetics Academic Medical Center – University of Amsterdam
Meibergdreef 9 1105 AZ Amsterdam The Netherlands

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Neuroblastoma is an embryonal tumour of the peripheral sympathetic nervous system (SNS). One of the master regulator genes for peripheral SNS differentiation, the homeobox transcription factor \textit{PHOX2B}, is mutated in familiar and sporadic neuroblastomas. Here we report that inducible expression of \textit{PHOX2B} in the neuroblastoma cell line SJNB-8 down-regulates \textit{MSX1}, a homeobox gene important for embryonic neural crest development. Inducible expression of \textit{MSX1} in SJNB-8 caused inhibition of both cell proliferation and colony formation in soft agar. Affymetrix micro-array and Northern blot analysis demonstrated that \textit{MSX1} strongly up-regulated the Delta-Notch pathway genes \textit{DLK1}, \textit{NOTCH3}, and \textit{HEY1}. In addition, the proneural gene \textit{NEUROD1} was down-regulated. Western blot analysis showed that \textit{MSX1} induction caused cleavage of the NOTCH3 protein to its activated form, further confirming activation of the Delta-Notch pathway. These experiments describe for the first time regulation of the Delta-Notch pathway by \textit{MSX1}, and connect these genes to the \textit{PHOX2B} oncogene, indicative of a role in neuroblastoma biology. Affymetrix micro-array analysis of a neuroblastic tumour series consisting of neuroblastomas and the more benign ganglioneuromas showed that \textit{MSX1}, \textit{NOTCH3} and \textit{HEY1} are more highly expressed in ganglioneuromas. This suggests a block in differentiation of these tumours at distinct developmental stages or lineages.

Abstract

Neuroblastoma is an embryonal tumour of the peripheral sympathetic nervous system (SNS). One of the master regulator genes for peripheral SNS differentiation, the homeobox transcription factor \textit{PHOX2B}, is mutated in familiar and sporadic neuroblastomas. Here we report that inducible expression of \textit{PHOX2B} in the neuroblastoma cell line SJNB-8 down-regulates \textit{MSX1}, a homeobox gene important for embryonic neural crest development. Inducible expression of \textit{MSX1} in SJNB-8 caused inhibition of both cell proliferation and colony formation in soft agar. Affymetrix micro-array and Northern blot analysis demonstrated that \textit{MSX1} strongly up-regulated the Delta-Notch pathway genes \textit{DLK1}, \textit{NOTCH3}, and \textit{HEY1}. In addition, the proneural gene \textit{NEUROD1} was down-regulated. Western blot analysis showed that \textit{MSX1} induction caused cleavage of the NOTCH3 protein to its activated form, further confirming activation of the Delta-Notch pathway. These experiments describe for the first time regulation of the Delta-Notch pathway by \textit{MSX1}, and connect these genes to the \textit{PHOX2B} oncogene, indicative of a role in neuroblastoma biology. Affymetrix micro-array analysis of a neuroblastic tumour series consisting of neuroblastomas and the more benign ganglioneuromas showed that \textit{MSX1}, \textit{NOTCH3} and \textit{HEY1} are more highly expressed in ganglioneuromas. This suggests a block in differentiation of these tumours at distinct developmental stages or lineages.
1. Introduction

Neuroblastoma is a tumour of early childhood, mostly diagnosed before the age of two years. It is a tumour of the peripheral sympathetic nervous system (SNS) with primary tumour sites in the adrenal medulla and along the SNS side chain [1-3]. Neuroblastoma belongs, together with ganglioneuroma and ganglioneuroblastoma, to the group of neuroblastic tumours. There is evidence for a differentiation gradient between the subtypes from immature to more mature tumours [4]. Neuroblastoma is the most undifferentiated subtype of the neuroblastic tumours. It usually consists of small, round neuroblast-like tumour cells. Ganglioneuroma is the most differentiated neuroblastic tumour, consisting of clusters of ganglion cells surrounded by a dense stroma of Schwann cells. Ganglioneuroblastoma is an intermixed form of neuroblastoma and ganglioneuroma [5]. Neuroblastoma occasionally differentiates into ganglioneuroblastoma and further to ganglioneuroma, while progression of ganglioneuromas and ganglioneuroblastomas towards neuroblastoma is very rare [4, 5]. Ganglioneuromas are invariably benign. Children with stage 1 or 2 neuroblastomas have an excellent prognosis, while stage 3 and 4 neuroblastomas are associated with a poor prognosis. Stage 4S neuroblastomas metastasize but usually go into spontaneous regression [2].

Neuroblastic tumours are derived of the sympatho-adrenal lineage of the peripheral SNS. The SNS is derived from the neural crest. The neural crest is a transient cell population formed during neurulation under influence of BMP, FGF, Wnt and Notch signalling from surrounding tissue [6-10]. The early neural crest cells subsequently express regulatory gene families like Dlx, Pax, Zic and Msx, which specify neural crest cell fate. Finally, individual cells delaminate from the neural crest, migrate through the body, and by a series of committing cell divisions form a number of different tissues including the SNS [7, 10]. The sympatho-adrenal lineage of the peripheral SNS gives rise to specialized cell types of the sympathetic side chain ganglia, the sympathetic paraganglia and the adrenal medulla [11].

It is unknown from what cell type and at which embryonal stage of SNS development neuroblastic tumours arise. The histopathological and clinical heterogeneity of neuroblastic tumours suggest that the different subtypes reflect a block in differentiation at various stages of development. Only a few genes with a causative role in neuroblastoma pathogenesis have been identified. MYCN is amplified in 20% of tumours [12, 13]. Furthermore, rare amplifications of ALK, CCND1, CDK4, MDM2, and MEIS1 have been found, as well as deletion of PTPRD [14-20]. Recently, we and others reported recurrent (but rare) mutations in the PHOX2B gene in sporadic neuroblastoma [21-23]. PHOX2B was also found as the first predisposing gene to hereditary neuroblastic tumours; PHOX2B germline mutations occur in some, but not all, pedigrees with familial neuroblastoma [23-27].

The PHOX2B transcription factor plays a key role in the early differentiation of the sympatho-adrenal lineage from neural crest cells. PHOX2B null-mutant mice fail to form autonomic ganglia or functional adrenal medullas and do not produce...
Figure 1: PHOX2B and MSX1 mRNA expression in neuroblastic tumours

Affymetrix micro-array analysis of PHOX2B and MSX1 mRNA expression in 96 neuroblastic tumour samples (13 ganglioneuromas and 83 neuroblastomas) using the Affymetrix HG-U133 Plus 2.0 microarray. The validity and specificity of the Affymetrix probes for MSX1 and PHOX2B was checked using TranscriptView (see Materials and Methods). In addition, the signals for MSX1 and PHOX2B detected on Northern blots of cell line time-course experiments correlated well with the expression values obtained with Affymetrix profiling (see Figure 2). We concluded that the PHOX2B and MSX1 Affymetrix expression values were very dependable. (A) PHOX2B mRNA expression in neuroblastic tumours: Upper graph: visual representation of PHOX2B expression in all 96 tumours. MSX1 expression values for each tumour are visualized on the vertical axis with a blue rectangle. Track below graph indicates tumour pathology: green ("GN") and red ("NB") rectangles for ganglioneuromas and neuroblastomas, respectively. Lower graph: bar plot of the average PHOX2B expression values detected in ganglioneuromas vs. neuroblastomas. Standard errors are indicated with vertical lines. (B) PHOX2B and MSX1 mRNA expression correlation in neuroblastic tumours: Visual representation of PHOX2B and MSX1 expression in all 96 tumours, ranked on the horizontal axis from left to right according to their PHOX2B expression. PHOX2B and MSX1 expression values for each tumour are visualized with red and blue circles, respectively. Further details as in Figure 1A.
MSX1 activates the Delta-Notch pathway

nor-adrenalin due to insufficient proliferation and increased cell death of sympatho-adrenal progenitor cells. Also, expression of tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH), key enzymes of the nor-adrenalin synthesis route were largely absent in the PHOX2B null-mutant mice [28, 29]. Thus PHOX2B activity is an essential determinant of the vertebrate noradrenergic phenotype. However, it is unknown how PHOX2B regulates normal differentiation of the sympatho-adrenal lineage and how mutations contribute to neuroblastoma pathogenesis.

To gain more insight into the PHOX2B downstream target gene network in neuroblastoma we analyzed the mRNA expression in 96 neuroblastic tumours by Affymetrix micro-arrays. PHOX2B expression appeared to have an inverse correlation with MSX1, a homeobox transcription factor involved in early neural crest development. Regulation of MSX1 expression by PHOX2B was confirmed in a neuroblastoma cell line with inducible PHOX2B expression. To study the role of MSX1 in neuroblastoma, we constructed a neuroblastoma cell line capable of inducible MSX1 expression. MSX1 appeared to control the expression of key genes of the Delta-Notch pathway, thereby identifying a novel connection between this important differentiation route and the PHOX2B gene.

Figure 2: PHOX2B regulates MSX1 expression in the SJNB-8 cell line

Affymetrix micro-array and Northern blot analysis of PHOX2B and MSX1 expression in the SJNB-8-TetR-PHOX2B cell line. PHOX2B expression was induced with doxycycline, total RNA was isolated during a time course experiment at the time points indicated after addition of doxycycline (+ dox). (A) Affymetrix micro-array analysis. Visual representation of MSX1 expression values measured. Time points analyzed were 0, 8, 24, 48, and 96 hours after addition of doxycycline. The Affymetrix analysis was performed on three independent time course experiments. No significant MSX1 expression changes were found in time-course experiments of mock-transfected SJNB-8-TetR cell lines (data not shown). (B) Northern blot analysis. 20 μg of total RNA from the experiments in panel A was used to prepare a Northern blot. The blot was hybridized with radio-active probes specific for PHOX2B and MSX1 mRNA. Ethidium bromide staining of the 28S rRNA band was used as a loading control.
2. Materials and Methods

2.1 Generation of inducible cell lines
Neuroblastoma cell line SJNB-8 [30] was a gift from the St. Jude Children’s Research Hospital (Memphis, TN, USA). SJNB-8 cells were grown in high-glucose DMEM (Dulbecco’s Modified Eagle’s Medium) without sodium-pyruvate with pyridoxine-HCl (Gibco 41965-039, Invitrogen, Breda, The Netherlands), supplemented with 10 % heat-inactivated foetal calf serum (Gibco 10106), 2 mM L-Glutamine (ICN 1680149, Cleveland, OH, USA), 1 x MEM (Minimal Non-Essential Amino Acids, Gibco 11140-035) and 10 U penicillin/10 μg streptomycin (Sigma P0781, St. Louis, MO, USA) per ml. All cells were maintained at 37 °C, in a humidified atmosphere containing 5% CO2. For the generation of SJNB-8 cell lines capable of tetracycline/doxycycline-regulated transgene expression, the T-Rex system (Invitrogen) was used. SJNB-8 cells were transfected with the pcDNA6/TR vector (Invitrogen V1025-20, Blasticidin resistance) encoding the tetracycline repressor behind a constitutive CMV promoter, using Lipofectamine 2000 (Invitrogen 11668). Clones were selected by limited dilution using 5 µg/ml Blasticidin-S (Invitrogen R210), resulting in the isolation of SJNB-8-TetR. Full length cDNAs of PHOX2B (encoding nucleotides 361-1305 of RefSeq NM_003924) and MSX1 (encoding nucleotides 236-1147 of RefSeq NM_002448) were cloned into pcDNA4/TO (Invitrogen V1030-20, Zeocin resistance) behind a tetracycline/doxycycline-inducible CMV promoter. The pcDNA4/TO cDNA constructs were transfected into SJNB-8-TetR using Lipofectamine 2000. Clones were selected by limited dilution using 5 µg/ml Blasticidin S and 50 µg/ml Zeocin (Invitrogen R250), resulting in the isolation of several SJNB-8-TetR-PHOX2B, and SJNB-8-TetR-MSX1 clones, respectively. The expression levels of MSX1 and PHOX2B were assayed at 48 h after addition of 50 ng/ml doxycycline (ICN 195044) by Western blotting and Northern blotting, respectively. Only clones that showed inducible expression of the transgene, as well as tight control of transgene expression by doxycycline were used in this study.

![Figure 3: Inducible expression of MSX1 in the SJNB-8 cell line](image)

Western blot analysis of MSX1 expression in the SJNB-8-TetR-MSX1 cell line (clones K2 and K5). MSX1 expression was induced with doxycycline, and total protein was isolated during a time course experiment at the indicated time points after addition of doxycycline (“+ dox”). 10 µg total protein was used to prepare a Western blot. The blot was probed with MSX1 antiserum, subsequent probing of the blot with β-actin antiserum was used as a loading control.
Figure 4: MSX1 expression causes growth retardation in the SJNB-8 cell line
Growth analysis of SJNB-8-TetR parental and SJNB-8-TetR-MSX1 cell lines using a modification of the NIH/3T3 protocol. (A) Growth analysis of SJNB-8-TetR and SJNB-8-TetR-MSX1 (clones K2 and K5) cells incubated without or with doxycycline ("- dox" and "+ dox", respectively) throughout the experiment. At the indicated time points, cells were counted and transferred to a fresh plate (see Materials and Methods). (B) Growth analysis of SJNB-8-TetR-MSX1 (clone K5) cells upon removal of doxycycline after 15 days of presence, or treated without or with doxycycline during the whole experiment (31 days). Depicted is one representative experiment for clone K5, experiments with clone K2 were similar (data not shown). Experiments were performed twice, in triplicate. Standard errors are represented by vertical lines.
2.2 Growth analysis and soft agar assay

Growth analysis was performed according to a modified NIH/3T3 protocol. Cells were counted using a Z1 Coulter Counter (Beckman Coulter, Mijdrecht, The Netherlands) according to the standard protocol. Cells were seeded at equal density and grown for 3 to 4 days. Subsequently, all cells were released by trypsinisation, counted and replated at the original density. This was continued for 21 or 31 days for Figures 4A and 4B, respectively. For the soft agar assay, SJNB-8-TetR-MSX1 cells (K5) were suspended in 0.4 % Noble Agar (Difco 21430, Becton Dickinson, Sparks, MD, USA) in complete culture medium at 5,000 cells/ml. 1 ml of this suspension was spread on 1 ml of 0.5 % agar in medium in 6 well dishes. In cells indicated with “+ dox”, MSX1 expression was induced by the presence of doxycycline (100 ng/ml final concentration) in the seeding medium, and medium covering the agar was refreshed every 2-3 days with fresh doxycycline-containing medium. Cells indicated with “- dox” were treated similarly, but without added doxycycline. After 21 days, cells were stained with methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma M2128, St. Louis, MO, USA), and colonies were counted using NIH ImageJ 1.37v software (http://rsb.info.nih.gov/ij/).

2.3 RNA isolation and Northern blot analysis

Total RNA was isolated from harvested cells according to the lithium chloride-urea method as described in [31] using three phenol-chloroform extractions followed by a single chloroform extraction, and subsequently precipitated at -20 °C in 70% ethanol-0.1 M sodium acetate (pH 5.2). RNA was resuspended in water. For Northern blot, 20 µg total RNA was electrophoresed through a 0.8% (w/v) agarose gel (Agarose Type IV, Sigma A3643) containing 6.7% (v/v) formaldehyde and blotted onto Hybond-N membrane (Amersham Biosciences RPN 203 N, Amersham, UK) overnight by capillary transfer in 16.9 x SSC and 5.7% formaldehyde. Membranes were dried, cross linked using 1200 W in a UV cross linker (Hoefer, San Francisco, CA, USA), and baked at 80 °C for 1 hour. Probe DNA (50 ng) was radio labelled using the random hexamer labelling protocol by [32] and deoxycytidine 5'-α32P-triphosphate (Amersham Biosciences AA0074). Non-incorporated label was removed using Sephadex G50 column chromatography (Pharmacia), remaining radio labelled probe was heat-denatured, and added to hybridization medium to typically 5 • 106 cpm/ml. Blots were hybridized according to a Church and Gilbert protocol [33], and subsequently exposed during 4-14 days to Amersham Hyperfilm MP (RPN 7 K) using double intensifying screens at -70 °C. The probes for endogenous and transgene MSX1 (encoding nucleotides 1316-1523 and 241-411 of the MSX1 RefSeq NM_002448, respectively) were generated using PCR with primers 5’-ccaaaaagttggctggaagag-3’ and 5’-cgattttcgcgtttct-3’ and 5’-cggtgtaccccgatgactttctgctttggactcttg-3’ and 5’-aggacgaaggggacacctt-3’, respectively, on neuroblastoma cDNA. Similarly, the NEUROD1 probe was produced with primers 5’-gaagagttggctggaagag-3’ and 5’-tgctcagtcttcggactctgccactctcg-3’ (encoding nucleotides 376-705 of the NEUROD1 RefSeq NM_002448). The PHOX2B, DLK1, NOTCH3 and HEY1 probes were described previously [21, 34]. All probes were verified by DNA sequencing before hybridisation.
2.4 Protein isolation and Western blot analysis
Cells were washed with PBS and lysed using RIPA (Radio immuno-precipitation buffer; 150 mM NaCl, 1.0% (v/v) NP40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, in 50 mM Tris-HCl (pH 8.0)). Protein concentrations were determined using the DC Protein assay kit I (Biorad 500-0116, Veenendaal, the Netherlands). Total protein (10 µg) in 1 x Laemmli sample buffer (4% (w/v) SDS, 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue and 125 mM Tris-HCl, (pH 6.8) supplemented with 10% (v/v) 2-mercaptoethanol was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to an Immobilon-P PVDF membrane (IPVH00010, Millipore, Amsterdam, the Netherlands) by electro-blotting (in 20% (v/v) methanol standard, in 10% (v/v) methanol for NOTCH3 blots), and blocked for 1 h. Membranes were incubated overnight at 4 °C with primary antiserum following incubation with a horseradish peroxidase conjugated secondary antibody for 1 h and developed using enhanced chemiluminescence (ECL RPN2132, GE Healthcare, Eindhoven, the Netherlands). Blocking solutions were 5% (w/v) non-fat dry milk/TBS/ 0.2% (v/v) Tween-20 for MSX1 and β-actin, and 5% (v/v) rabbit serum/TBS/ 0.1% (v/v) Tween-20 for NOTCH3 antisera. Antisera against NOTCH3 (M-20) and MSX1 (E-20) were purchased from Santa Cruz (Santa Cruz, CA, USA), β-actin antiserum was purchased from Abcam (AB6276, Cambridge, UK).

2.5 Affymetrix micro-array analysis
Snap frozen neuroblastic tumours were cut in a microtome at - 25°C and concurrently documented with haematoxylin/eosin staining of the samples throughout the cutting procedure to ensure > 95 % tumour cell content prior to RNA extraction. The sections were pathology reviewed by two pathologists and classified according to [5]. RNA concentration was determined using a NanoDrop (NanoDrop Technologies, Wilmington, DE, USA) and RNA quality assessed using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Fragmentation of cRNA, hybridization to HG-U133 Plus 2.0 microarrays and scanning was carried out according to the manufacturer’s protocol (Affymetrix Inc., Santa Barbara, CA, USA) at the MicroArray Department of the University of Amsterdam. Intensity values and the accompanying p-values were assigned to probe-sets with GeneChip® Operating Software (GCOS) using the MASS5.0 algorithm (Affymetrix Inc., Santa Barbara, CA, USA). Affymetrix probe-sets were evaluated and selected using the TranscriptView tool for the alignment of NCBI mRNA and EST sequences along the human genome that allows visualization of the structure of a gene locus or chromosomal domain ([35] and http://bioinfo.amc.uva.nl/human-genetics/transcriptview). The Affymetrix probe-sets selected were 207009_at (PHOX2B), 205932_s_at (MSX1), 203238_s_at (NOTCH3), 218839_at (HEY1), 209560_s_at (DLK1), and 206282_at (NEUROD1). The SJNB-8-TetR-MSX1 micro-array data have been deposited for public access in a MIAME-compliant format through the GEO database at the NCBI website under number GSE9339.
3. Results

**PHOX2B regulates the MSX1 homeobox transcription factor in neuroblastoma cells**

Knowledge about the molecular pathways regulated by the PHOX2B transcription factor in neuroblastic tumours is scarce. We therefore analyzed the PHOX2B expression in Affymetrix micro-array data of a series of 83 neuroblastomas and 13 ganglioneuromas. PHOX2B showed a significant higher expression in the neuroblastoma tumours than in the ganglioneuroma tumours (Figure 1A; \( p = 1.5 \times 10^{-8} \); Mann-Whitney U test).

To investigate the downstream signalling pathways of PHOX2B, we analyzed the Affymetrix tumour data for transcription factors that showed a significant correlation with PHOX2B expression. A total of 233 transcription factors demonstrated a positive or inverse correlation with PHOX2B (2log Pearson correlation > 0.4 or < -0.4, \( p < 10^{-4} \)). A complete analysis of this putative PHOX2B signalling network will be discussed elsewhere [Versteeg et al., manuscript in preparation]. For this study, we concentrated on transcription factors known to function in neural crest development. Expression of the MSX1 homeobox gene appeared to have a significant inverse correlation to PHOX2B expression \( (r = -0.405, p = 4.2 \times 10^{-5}; \text{Pearson correlation}) \). Like PHOX2B, MSX1 has a clear role in the formation of neural crest. MSX1 is expressed early in neural development in the neural folds, as well as in the pre-migratory and migratory neural crest cells [6, 8, 10]. MSX1 was most strongly expressed in ganglioneuromas (Figure 1B). We therefore asked whether MSX1 expression might be down-regulated by PHOX2B.

We used the SJNB-8 neuroblastoma cell line to construct a clone with tetracycline/doxycycline inducible PHOX2B transgene expression (SJNB-8-TetR-PHOX2B). PHOX2B expression was induced in this clone by doxycycline, and a time course experiment was performed. RNA isolated at various time points after the addition of doxycycline was analyzed by Affymetrix micro-arrays. Figure 2A shows down-regulation of MSX1 expression values after PHOX2B induction in three independent time course experiments. The induction of PHOX2B and subsequent down-regulation of MSX1 was confirmed by Northern blot analysis of the time-course experiment (Figure 2B). MSX1 expression is reduced within two hours after the induction of PHOX2B and remains low during the 8 days of the experiment, indicating stringent control of MSX1 expression by PHOX2B.

**3.1 MSX1 expression inhibits cell proliferation and colony formation**

To further analyze the PHOX2B-MSX1 axis in neuroblastoma, we generated SJNB-8 cell line clones capable of tetracycline/doxycycline inducible MSX1 transgene expression (SJNB-8-TetR-MSX1). Several clones with non-leaky, inducible MSX1 expression were isolated, and clones K2 and K5 were used for further analyses. Western blot analysis of both clones showed that MSX1 protein, normally undetectable in SJNB-8, was induced within two hours after doxycycline addition (Figure 3).
MSX1 activates the Delta-Notch pathway

In a proliferation-assay, induction of MSX1 expression in the SJNB-8-TetR-MSX1 K2 and K5 clones resulted in severe growth inhibition, as compared to the non-induced clones. The growth inhibition was a specific effect of MSX1 expression since it was not observed in the SJNB-8-TetR parental cell line upon treatment with doxycycline (Figure 4A). The growth suppression could be reversed upon removal of doxycycline, which resulted in complete disappearance of MSX1 protein (data not shown). This treatment restored the cell proliferation rate to that of non-treated cells (Figure 4B). MSX1 expression also reduced the colony-forming capacity of SJNB-8 cells in soft agar by 50%, indicating that MSX1 represses anchorage independent growth (Figures 5A and 5B).

The induction of MSX1 expression in other experimental systems has been reported to trigger apoptosis or cell cycle arrest (see Discussion). However, this was not observed in our clones: Western blot analysis did not show increased cleavage of the caspase substrate protein PARP after MSX1 induction (data not shown). FACS analysis did not reveal any difference in the distribution of the cell cycle (data not shown). MSX1 expression therefore appeared to slow down the proliferation of SJNB-8 cells, rather than to trigger apoptosis or cell cycle block.

As MSX1 was down-regulated by the PHOX2B oncogene and as MSX1 expression slowed down proliferation of neuroblastoma cells, we wondered whether MSX1 acts
as a tumour suppressor gene and shows inactivating mutations in neuroblastomas. Chromosomal DNA encoding the two MSX1 exons was sequenced in 73 neuroblastoma tumours and cell lines, but no mutations were detected, except for two putative SNPs (Table I). This seemed to preclude a role for MSX1 as classical tumour suppressor gene. As MSX1 maps in a region on chromosome band 4p16 that shows LOH in 15-20 % of neuroblastomas [36-39], we can not exclude that haplo-insufficiency for MSX1 plays a role in neuroblastoma.

3.2 Analysis of MSX1 downstream pathway in SJNB-8
To further elucidate the molecular pathways that are regulated via the PHOX2B-MSX1 axis, we performed Affymetrix micro-array analyses of RNA isolated from time course experiments of SJNB-8-TetR-MSX1 clones K2 and K5. RNA was isolated at 0 and 48 hours after addition of doxycycline for clone K2, and at 0, 24, 48, and 192 hours for clone K5. We used the R2 bio-informatical analysis program developed in our lab

Figure 6: MSX1 regulates genes of the Delta-Notch pathway in the SJNB-8 cell line
Affymetrix micro-array and Northern blot analysis of Delta-Notch pathway genes in the SJNB-8-TetR-MSX1 cell line (clones K2 and K5). MSX1 expression was induced with doxycycline, and total RNA was isolated during a time course experiment at the time points indicated after addition of doxycycline (“+ dox”). (A) Affymetrix micro-array analysis. Visual representation of NOTCH3, HEY1, DLK1, and NEUROD1 gene expression values measured. Time points analyzed were 0 and 48 hours after addition of doxycycline for clone K2, and 0, 24, 48, and 192 hours for clone K5. No significant expression changes were found for these genes in time-course experiments of mock-transfected SJNB-8-TetR cell lines (data not shown). (B) Northern blot analysis. The blots were hybridized with radio-active probes specific for MSX1, NOTCH3, HEY1, DLK1, and NEUROD1 mRNA. Only the results for clone K5 are shown. Other details as in Figure 2B.
MSX1 activates the Delta-Notch pathway

Table 1: Putative MSX1 SNPs in 73 neuroblastoma tumours and cell lines
MSX1 SNPs detected by genomic DNA sequencing of 73 neuroblastoma tumours and cell lines. Nucleotide and codon indicate the nucleotide and amino acid position, respectively, in the MSX1 RefSeq NM_002448. NCBI dbSNP details can be found on http://www.ncbi.nlm.nih.gov/SNP/ under their refSNP ID.

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[Koster et al., manuscript in preparation] to search for genes that are regulated by MSX1. We identified 112 genes with a significant up- or down-regulation upon MSX1 induction in both clones (cut-off values 2logfold regulation of at least 0.8; minimal expression value of 60). In our further analysis, we focused on genes involved in neural crest development. Several of the MSX1-regulated genes belonged to the Delta-Notch signalling network, which is implicated in the development of the neural crest and the SNS [40, 41]. The important Delta-Notch pathway genes NOTCH3, HEY1, and DLK1 showed mRNA induction within 48 hours after MSX1 expression (Figure 6A). Northern blot analysis confirmed the strong induction of all these genes (Figure 6B).

Delta, Notch and Hey family genes function in a sequential way in the Delta-Notch pathway. Delta-family proteins are trans-membrane proteins that can activate Notch receptors on neighbouring cells. This results in cleavage of the intracellular domain of Notch (Notch-IC) and translocation to the nucleus where it functions as transcription factor [42-44]. A major group of NOTCH transcriptional targets is formed by the HES family of transcription factors, that includes the HEY genes [45-47]. The Northern

SJNB8-TetR-MSX1 K5

Figure 7: MSX1 activates Delta-Notch signalling in the SJNB-8 cell line
Western blot analysis of MSX1 expression and NOTCH3 activation in the SJNB-8-TetR-MSX1 cell line (clones K2 and K5). The blots were incubated with MSX1 or NOTCH3 antisera. Only the results for clone K5 are shown. Molecular weight (in kDa) is indicated on the left side. Arrows at the right side point to the full length Notch3 (~ 280 kDa), and IC domain (~ 95 kDa). No significant increase in NOTCH3 (full length or IC) expression was observed during a time course experiment with the SJNB8-TetR parental cell line (data not shown). Other details are as in Figure 3.
blot analyses of the time-course experiments shows a complex induction pattern of
the three Delta-Notch pathway genes. NOTCH3 expression starts to increase about
24 h after MSX1 induction, but full transcription activation is not seen until about day
4 (Figure 6B). Surprisingly, induction of HEY1 expression is already observed before
the rise in NOTCH3 mRNA expression. The Northern blot analysis shows onset
of HEY1 induction within 2 hours after MSX1 induction (Figure 6B). Peak levels of
HEY1 are then reached within the next 24 hours. DLK1 (Delta-like 1 homolog) is a
human Delta family gene, but a function as Notch-ligand has not been unequivocally
established for Dlk1 [42, 48, 49]. Up-regulation of DLK1 mRNA levels was first
evident 48 hours after MSX1 induction (Figure 6A).

3.3 Activation of the Delta-Notch pathway by MSX1
To further investigate the paradoxical finding that HEY1 mRNA induction was
evident prior to significant NOTCH3 mRNA accumulation, we analyzed the kinetics
of MSX1 time-courses at the protein level. Western blot analysis of MSX1 induction
showed MSX1 protein expression already 4 hours after induction (Figure 7). We
subsequently analyzed the time-course for expression of the NOTCH3 protein. Of
special interest was appearance of the cleaved ~ 95 kDa NOTCH3-IC fragment
(full-length NOTCH3 protein is ~ 280 kDa in size [50]). NOTCH3-IC was hardly
detectable before induction of MSX1 at t=0, but already 4 hours later, a stronger
NOTCH3-IC signal became visible. The NOTCH3-IC protein accumulated during
the next 16 hours. Accumulation of the full length NOTCH3 protein could also be
seen. The relative amounts of NOTCH3 full-length and IC fragment show that most
of the NOTCH3 protein is cleaved to its active form. These data strongly suggest
that the appearance of NOTCH3-IC precedes the transcriptional activation of the
NOTCH3 gene and the accumulation of NOTCH3 mRNA. SJNB-8 cells have a low
but detectable NOTCH3 expression (Figure 6B). Induction of MSX1 expression in
the SJNB-8 clones evidently leads to a swift cleavage of NOTCH3 receptor protein
already present in the cell, causing a fast activation of NOTCH3, and –as seems
evident by the appearance of HEY1 mRNA within 2 hours- of its downstream target
genes.

MSX1 therefore not only mediates a relatively late transcriptional activation of
NOTCH3, but also a faster activation at the protein level by triggering cleavage of
NOTCH3-IC through an as yet unknown mechanism. Binding of Delta-family proteins
to NOTCH3 might trigger NOTCH3-IC cleavage in the SJNB-8 clones expressing
MSX1 [42-44, 48, 49], but it is unlikely that the observed transcriptional induction
of DLK1 by MSX1 is responsible for NOTCH3 cleavage, as the first increase of DLK1
mRNA levels is not observed before 8-24 hours after MSX1 activation.

3.4 Induction of the proneural NEUROD1 gene
The appearance of a considerable amount of NOTCH3-IC fragment during the MSX1
time-course and the induction of HEY1 mRNA levels suggest that the Delta-Notch
pathway is functionally activated. One of the well-established roles of Notch activity
in neural crest cell fate specification is the inhibition of neuronal differentiation and
the promotion of other nervous system cell fates [40]. This inhibitory role of Notch
MSX1 activates the Delta-Notch pathway

is invariably accompanied and mediated by the repression of “proneural genes” [40, 41, 51]. The proneural genes encode a group of basic helix-loop-helix (bHLH) transcription factors that are essential for neuronal cell fate specification [51]. We therefore investigated the expression of proneural genes in the SJNB-8-TetR-MSX1 time course experiments. The proneural gene NEUROD1 [52] appeared to be repressed after MSX1 induction in clones K2 and K5 (Figure 6A). Northern blot analysis confirmed that NEUROD1 expression was reduced within 2 hours after MSX1 induction and remained low for at least 4-6 days (Figure 6B).

3.5 Expression patterns in neuroblastic tumours
The strong regulation of NOTCH3, HEY1, DLK1, and NEUROD1 by experimental MSX1 induction in the SJNB-8 cell line raised the question whether these Delta-Notch pathway genes may play a role in neuroblastoma biology. We therefore analyzed the expression of these genes in the Affymetrix micro-array tumour database described above. NOTCH3 and HEY1 showed a higher expression in the benign ganglioneuroma

![Figure 8: HEY1 expression in neuroblastic tumours](image)

Affymetrix micro-array analysis of HEY1 expression in 96 neuroblastic tumour samples (13 ganglioneuromas and 83 neuroblastomas). Upper graph: Visual representation of HEY1 expression in all 96 tumours. Lower graph: bar plot graph of average HEY1 expression values detected in ganglioneuromas vs. neuroblastomas. Other details as in Figure 1A.
tumours, and lower expression in the less differentiated neuroblastoma samples. Expression of NOTCH3 was on average almost 2-fold higher in ganglioneuroma (p= 4.0 • 10^{-3}; Mann-Whitney u test, data not shown). HEY1 showed a 3-fold higher average expression in ganglioneuromas than in neuroblastomas (Figure 8, p= 1.3 • 10^{-8}; Mann-Whitney u test). No significant differences in expression were observed for DLK1 or NEUROD1 (data not shown).

In conclusion, NOTCH3, HEY1 and MSX1 are more highly expressed in ganglioneuromas, while PHOX2B is highly expressed in neuroblastoma. The significant correlations of these genes with the histological differentiation grade of the neuroblastic tumours suggest an important role for these genes and their pathways in neuroblastoma biology.

4. Discussion

The rare but recurrent occurrence of PHOX2B mutations in both sporadic and hereditary neuroblastic tumours [21-27] indicates that PHOX2B controls essential pathways in neuroblastoma pathogenesis. In this study we showed that the MSX1 homeobox gene was down-regulated after induction of PHOX2B expression. MSX1 is a transcription factor with an important function in early neuronal development [6, 8, 10]. We therefore further followed this line of cellular signalling by studying inducible expression of MSX1. MSX1 on its turn was found to induce the expression of three major genes of the Delta-Notch neural differentiation pathway, NOTCH3, HEY1, and DLK1 and down-regulate expression of the proneural gene NEUROD1. Analysis of the kinetics of induction at the mRNA and protein level suggested an early appearance of the activated NOTCH3-IC fragment. MSX1 therefore not only induced NOTCH3 mRNA expression, but also caused NOTCH3 activation at the protein level by cleavage of the NOTCH3-IC fragment. This most likely explains the strong induction of HEY1, as the HES and HEY family members are known transcriptional targets of NOTCH. The HES and HEY genes on their turn encode helix-loop-helix transcription factors known to inhibit neuronal differentiation by down-regulation of proneural genes. Accordingly, we detected a clear down-regulation of the proneural gene NEUROD1.

These observations still leave a number of questions. How does MSX1 induce the early cleavage of the NOTCH3 protein? Does DLK1 function as the ligand for NOTCH3? Furthermore, clones capable of inducible expression of e.g. NOTCH3 will be required to confirm that HEY1 and NEUROD1 are downstream of NOTCH3 in neuroblastoma cells. The most important finding of this study is however, that a molecular pathway emerges from PHOX2B, via MSX1 to the Delta-Notch signalling route and ultimately inhibition of proneural gene expression. Potentially, wild-type PHOX2B expression in neuroblastoma cells could permit NEUROD1 expression. A further evaluation of this novel pathway will be important to understand the role of PHOX2B in neuroblastoma differentiation and pathogenesis. Study of the role of
PHOX2B and MSX1 in embryonal development will aid in this comprehension.

The role of MSX1 and MSX homologues in embryonal development has been studied in a series of model organisms. Studies in mouse showed that embryonic MSX1 expression is commonly found at inductive borders of epithelial and mesenchymal tissues, indicating that MSX1 functions in early responses to diffusible factors (like BMP, FGF, and Wnt) that control cell growth and cell fate specification [53-55]. MSX1 expression was found to be important for the early development of several embryonic structures in mouse; e.g. neural crest, heart, mouth and face, and limb buds [54, 56, 57]. In addition, it has been shown that MSX1 is essential for neural crest specification [58, 59]. Manipulation of msh, the Drosophila homologue of MSX1, showed that msh was necessary for dorsoventral neuroblast cell fate specification in the developing nervous system: Msh loss of function resulted in a dorsal-to-ventral fate switch, but no overt cell death. In contrast, msh gain of function led to decreased development of midline and ventral neuroblasts as well as to apoptosis and faulty differentiation of these cells. Msh therefore seemed to function in repressing neuroblast ventral cell fate [60]. Functional studies of MSX1 in vertebrates also showed a crucial role for MSX1 in determining neuroblast cell fate in the embryonic nervous system. In MSX1 null-mutant mice, the dorsal midline, centre for dorsal-ventral patterning, was not formed, and neuroblasts switched from dorsal to ventral cell fate [61]. In Xenopus, MSX1 over-expression caused apoptosis and repression of neural crest cell differentiation [62]. In chicken finally, ectopic MSX1 expression resulted in increased apoptosis as well as in decreased cell cycle exit by the repression of terminal differentiation genes, including several proneural genes [63].

The conserved role for MSX1 in nervous tissue development therefore seems to be the repression of neural differentiation (see also [64]). A similar function of MSX1 was observed in other embryonal mouse tissues, as expression of MSX1 in embryonal bone and dentus [65], skeletal muscle [66, 67], limb buds [68], and mammary glands [69] inhibited or even reversed differentiation. The mechanism by which MSX1 inhibits differentiation in these studies is not fully appreciated. MSX1 has been shown to bind histone H1b and induce a repressed chromatin state in promoters of differentiation genes [67]. Hu et al. found that MSX1 could prevent terminal differentiation by blocking cell cycle exit, without influencing cell proliferation. MSX1-expressing cells thereby do not differentiate, and remain proliferative [69]. MSX1 transgene expression in HeLa (cervix), OVCAR3 (ovarian), and H1299 (lung) cancer cell lines caused cell cycle arrest and/or apoptosis [70, 71]. MSX1 supported p53 function in HeLa cell apoptosis and suppressed mouse xenograft growth of HeLa cells [71]. Also in other cancers therefore, MSX1 expression seems to be associated with slower cell growth, and increased apoptosis.

The induction of the Delta-Notch pathway activity as shown in our study therefore represents a new mechanism of action of MSX1. As a result, expression of the proneural gene NEUROD1 was inhibited. During neural crest cell fate specification, one of the actions of Notch is the inhibition of proneural genes, leading to reduced neuronal differentiation, and the induction of alternative nervous system cell fates.
Analysis of *NEUROD1* null mutant mice showed that *NEUROD1* expression is required for the differentiation of progenitor neurons in the brain, and loss of *NEUROD1* function caused massive neuron cell death [73]. Interestingly, *NEUROD1* gene expression has been shown to accompany neuronal differentiation of several human neuroblastoma cell lines [74-77]. In the Affymetrix micro-array tumour database described above, *NEUROD1* expression was significantly correlated with poor prognosis and *MYCN* amplification (results not shown), a paradoxical result for a neuronal differentiation gene. The exact mode of *NEUROD1* action in neuroblastoma cells can therefore not yet be described.

Neuroblastic tumours form a highly heterogeneous group of peripheral SNS malignancies. Understanding of the normal differentiation routes of the peripheral SNS might lead to an understanding of the different subtypes of neuroblastic tumours, as these may relate to specific developmental stages and lineages. Here we show that a gene that is clearly involved in neuroblastoma pathogenesis, *PHOX2B*, is connected to pathways active in early steps of SNS differentiation: both *MSX1* and Delta-Notch signalling are well studied in neural crest development. Our observation of Delta-Notch pathway regulation by *MSX1* was never described before and may explain part of the function of *MSX1* in normal development. The establishment of a signalling axis leading from *PHOX2B* via *MSX1* to Delta-Notch and proneural gene expression might offer new insights in neuroblastoma pathogenesis. The differential expression of several of the main players in this signalling cascade in ganglioneuromas versus neuroblastomas supports a role for these pathways in neuroblastic tumour biology.

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