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MSX1 induces the Wnt pathway antagonist genes DKK1, DKK2, DKK3, and SFRP1 in neuroblastoma cells, but does not block Wnt3 and Wnt5A signalling to DVL3

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ABSTRACT

Neuroblastoma is the most common extra-cranial solid childhood cancer; it arises from neural crest-derived cells of the sympathetic nervous system. The anomalous regulation of embryonic developmental pathways like Delta-Notch and Wnt has been implicated in aberrant cell growth and differentiation in many (childhood) tumours. We have previously found regulation of Delta-Notch pathway genes by the MSX1 neural crest development gene in a neuroblastoma cell line, and significant correlations between these genes in neuroblastic tumours. However, a clear role for the Wnt pathway in neuroblastic tumours has not yet been determined. We now analyze the complete spectrum of genes regulated by inducible expression of MSX1 in the SJNB8 neuroblastoma cell line using Affymetrix expression profiling. We show that MSX1 induces the expression of four different Wnt pathway inhibitor genes: Dickkopf 1-3 (DKK1-3) and secreted frizzled-related protein 1 (SFRP1), and provide evidence that high expression of two of these genes correlates with good prognosis. We were able to demonstrate that both the canonical Wnt3 and the alternative Wnt5A ligands are highly expressed in neuroblastic tumours and cell lines, and specifically activate the DVL3 Wnt co-receptor protein in SJNB8 neuroblastoma cells. These results suggest involvement of MSX1 in Wnt signalling and demonstrate activity of the more upstream Wnt pathway in neuroblastic cells.

1. Introduction

Neuroblastoma is the most common extra-cranial solid cancer in childhood; it arises from neural crest-derived cells of the sympathetic nervous system. Neuroblastomas account for ~15% of all paediatric cancer deaths. The tumours display a wide spectrum of clinical behaviour; some tumours regress spontaneously whereas others are highly malignant, with very poor prognosis despite intensive therapy [1]. The cause of neuroblastoma tumorigenesis remains largely unknown, since recurrent gene defects were identified in only about one-third of tumours. Amplification of the MYCN oncogene is found about 20% of tumours [1], while CCND1 is amplified in 3% of neuroblastomas [2]. Mutations in the PHOX2B [3,4] and ALK [5] genes are found in 4% and 8% of tumours, respectively.

Neuroblastomas belong to the group of neuroblastic tumours, which also include ganglioneuroblastomas, and ganglioneuromas. Ganglioneuromas are differentiated with an invariably good prognosis, while neuroblastomas have an undifferentiated morphology. Ganglioneuroblastomas are intermixed with a more positive prognosis [6,7]. The histological classification of neuroblastic tumours argues for a biological continuum of differentiation. This hypothesis is supported by the observation that malignant neuroblastoma tumours can occasionally transdifferentiate into ganglioneuroblastomas or ganglioneuromas. Conversely,

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specific (nodular) cases of ganglioneuroblastoma can progress to neuroblastoma [6–8]. Regulatory pathways that underlie these differentiation processes are therefore of special interest as potential targets for treatment.

The early formation of the neural crest, and the subsequent development, migration and terminal differentiation of neural crest-derived cells is regulated by at least three key pathways: the bone morphogenetic protein (BMP), the fibroblast growth factor (FGF), and Wnt signalling pathways [9–11]. Up-regulation of BMP in non-neural ectoderm triggers the neural crest differentiation program and expression of the MSX1 homeobox transcription factor in neural crest cells. MSX1 on its turn induces expression of BMP and Wnt in neural crest cells (reviewed in [12]). Regulation of Wnt genes by MSX was e.g. shown by loss of Wnt1 expression in MSX1/MSX2 double null-mutant mice, and by induction of Wnt1 in chick brain and lateral ectoderm following ectopic MSX1 expression [13]. While MSX1 induces BMP and Wnt, both pathways in turn upregulate MSX1 [12,14]. Hence, the MSX genes function as intermediate between the BMP and Wnt signalling pathways and may balance the two pathways to ensure proper differentiation of neural crest cells. In general, the MSX transcription factors fulfill this function by repressing target gene expression [12,15].

Embryonal oncogenesis is thought to occur by the disruption of the normal embryonic developmental program, thereby giving rise to aberrant cell growth and differentiation, and finally to cancer. In line with this, anomalous regulation of developmental pathways such as Delta-Notch and Wnt is involved in malignant growth in different paediatric tumours [16–18]. Both pathways play key roles in normal neural crest development and neuronal differentiation, and are interesting candidate pathways for neuroblastoma tumorigenesis [9–12,14]. Several studies have implied the Delta-Notch pathway in neuroblastoma pathogenesis [19–23]. Also the Wnt pathway has been investigated, but the role of Wnt signalling in neuroblastoma has remained elusive [8,24–29].

The PHOX2B homeobox transcription factor is exclusively expressed in the nervous system, and is crucial for differentiation of neural crest cells into sympathetic neurons and chromaffin cells [30,31]. PHOX2B can be mutated in both sporadic and familial neuroblastoma [3,4]. We recently showed that expression of a PHOX2B transgene silenced MSX1 expression in a neuroblastoma cell line. Accordingly, we observed an inverse correlation between PHOX2B and MSX1 expression in a panel of 110 neuroblastic tumours, with PHOX2B expression being highest in neuroblastomas and MSX1 expression peaking in ganglioneuroma and ganglioneuroblastoma [22].

We set out to identify MSX1 downstream target genes in neuroblastoma by Affymetrix expression analysis using time-course experiments of SJNB8 neuroblastoma cell line clones with inducible expression of an MSX1 transgene. We have previously reported activation of the Delta-Notch signalling pathway, inhibition of cell growth, and decreased anchorage-independence as a result of MSX1 expression [22]. Here we report that the MSX1 transcription factor induces expression of several inhibitors of the Wnt pathway. We also observed significant prognostic values for these Wnt inhibitors in neuroblastic tumours. We confirm earlier data on the low activity of the canonical β-catenin/TCF Wnt signalling pathway in neuroblastoma cell lines. However, many upstream Wnt and DVL genes were well expressed in neuroblastic tumours and cell lines, and we could show activation of DVL3 by the canonical Wnt3 and non-canonical Wnt5A ligands. These data for the first time show that upstream Wnt signalling is active in neuroblastoma cells, and that key genes in the pathway are regulated by the MSX1 neural crest differentiation gene.

2. Materials and methods

2.1. Generation of cell lines

The SJNB8 NB cell line was a gift from the St. Jude's Research Hospital (Memphis, TN, USA). It was derived from a metastatic NB tumour (N1108L) before treatment, and has MYCN amplification [32,33]. SJNB8 was chosen for MSX1 expression manipulation since it showed median MSX1 expression in a panel of 24 NB cell lines tested (results not shown), allowing both MSX1 over-expression and shRNA-mediated knock-down to levels comparable to those in other NB cell lines. Cell lines in this study were maintained in high-glucose DMEM without sodium-pyruvate with pyridoxine–HCl (Gibco 41965-039, Invitrogen, Breda, The Netherlands), supplemented with 10% heat-inactivated fetal calf serum (Gibco 10106), 2 mM L-Glutamine (ICN 1680149, Cleveland, OH), 1 × Minimal Non-Essential Amino Acids (Gibco 11140-035) and 10 U penicillin/10 μg streptomycin (Sigma P0781, St. Louis, MO) per ml. All cells were maintained at 37 °C, in a humidified atmosphere containing 5% CO_2. Generation of the SJNB8-TetR-MSX1 cell line clones capable of tetracycline/doxycycline-inducible MSX1 transgene expression (called SJNB8-MSX1 in this study) using the SJNB8-TetR parental cell line has been described previously [22].

2.2. RNA isolation, Affymetrix micro-array analysis and Northern blot analysis

RNA isolation, Affymetrix micro-array analysis, and Northern blot were performed as described previously [22]. All expression micro-array analysis in this study was performed on a genome-wide mRNA expression platform (Affymetrix HG-U133 Plus 2.0, Santa Clara, CA). The Affymetrix probe-sets used were selected using the TranscriptView application (http://bioinfo.amc.uva.nl/human-genetics/transcriptview), and are listed in Tables 1–3. Probes for Northern blot analysis were generated using RT-PCR on neuroblastoma cDNA with primers specific for MSX1 (5′-ccaaaaagtggctggaagag-3′ and 5′-cgatttctctg-cgcttttct-3′, producing a probe encoding nucleotides 1316–1523 of the MSX1 RefSeq NM_002448), DKK1 (5′-gacaactaccagccgtaccc-3′ and 5′-tgcatttggatagctggttt-3′, 386–973 of NM_012242), DKK2 (5′-agggcctgtcttgcaaagta-3′ and 5′-cactgcatttgtcacccatt-3′, 1409–1675 of NM_014421), DKK3 (5′-agcatgtactgccagtttgc-3′ and 5′-tcatact-catcggggacctc-3′, 705–1165 of NM_015881) and SFRP1