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Wnt Signalling Drives Context-Dependent Differentiation or Proliferation in Neuroblastoma[1]

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Abstract

Neuroblastoma is one of the commonest and deadliest solid tumours of childhood, and is thought to result from disrupted differentiation of the developing sympathoadrenergic lineage of the neural crest. Neuroblastoma exhibits intra- and intertumoural heterogeneity, with high risk tumours characterised by poor differentiation, which can be attributable to MYCN-mediated repression of genes involved in neuronal differentiation. MYCN is known to co-operate with oncogenic signalling pathways such as Alk, Akt and MEK/ERK signalling, and, together with c-MYC has been shown to be activated by Wnt signalling in various tissues. However, our previous work demonstrated that Wnt3a/Rspo2 treatment of some neuroblastoma cell lines can, paradoxically, decrease c-MYC and MYCN proteins. This prompted us to define the neuroblastoma-specific Wnt3a/Rspo2-driven transcriptome using RNA sequencing, and characterise the accompanying changes in cell biology. Here we report the identification of ninety Wnt target genes, and show that Wnt signalling is upstream of numerous transcription factors and signalling pathways in neuroblastoma. Using live-cell imaging, we show that Wnt signalling can drive differentiation of SK-N-BE(2)-C and SH-SY5Y cell-lines, but, conversely, proliferation of SK-N-AS cells. We show that cell-lines that differentiate show induction of pro-differentiation BMP4 and EPAS1 proteins, which is not apparent in the SK-N-AS cells. In contrast, SK-N-AS cells show increased CCND1, phosphorylated RB and E2F1 in response to Wnt3a/Rspo2, consistent with their proliferative response, and these proteins are not increased in differentiating lines. By meta-analysis of the expression of our 90 genes in primary tumour gene expression databases, we demonstrate discrete expression patterns of our Wnt genes in patient cohorts with different prognosis. Furthermore our analysis reveals interconnectivity within subsets of our Wnt genes, with one subset comprised of novel putative drivers of neuronal differentiation repressed by MYCN. Assessment of β-catenin immunohistochemistry shows high levels of β-catenin in tumours with better differentiation, further supporting a role for canonical Wnt signalling in neuroblastoma differentiation.

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Abbreviations: ALK, Anaplastic Lymphoma kinase; ATRA, all-trans-retinoic acid; BMP4, Bone morphogenetic protein 4; CCND1, Cyclin D1; EGF, Epidermal growth factor; EPAS1, Endothelial PAS Domain Protein 1; ERK, extracellular signal-regulated kinases; EMT, Epithelial-mesenchymal transition; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; MEK, Mitogen-activated protein kinase kinase; PBS, Phosphate-buffered saline; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; RB, retinoblastoma; RNAseq, RNA sequencing; Rspo2, R-Spondin-2; SDS-PAGE, sodium-dodecyl sulphate-polyacrylamide gel electrophoresis; TCF/Lef, T-cell factor/lymphoid Enhancer Binding Factor.

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Introduction

Neuroblastoma is responsible for approximately 15% of paediatric cancer deaths, with about 40% of patients considered to be high-risk cases with very poor prognosis [1,2]. Neuroblastoma is a biologically and clinically heterogeneous cancer arising from the sympathoadrenergic lineage of the neural crest [3]. Very recently, studies have demonstrated that tumours and cell-lines derived from them exhibit cellular heterogeneity based on enhancer usage and core regulatory transcriptional circuits [4,5]. The paradigmatic mechanism for disrupted differentiation in neuroblastoma is contingent on amplification of the *MYCN* proto-oncogene [6], with high levels of MYCN protein leading to direct repression of genes necessary for terminal differentiation in the sympathetic nervous system [7,8]. As well as *MYCN* amplification (MNA), high risk neuroblastomas have also been shown to elevate telomerase reverse transcriptase (*TERT*) expression through deregulatory genomic rearrangements [9,10]. Kinase pathways are also deregulated in neuroblastoma, including activating mutations of ALK [11], increased Akt signalling in stage 3 and 4 neuroblastoma [12], and relapsing neuroblastomas displaying mutations in the Ras-MAPK pathway [13,14].

Another oncogenic pathway reported to be involved in neuroblastoma, as well as many other cancers is canonical Wnt signalling. Here signalling is mediated by secreted Wnt ligands that are bound by membrane receptor complexes including Frizzled proteins and LRP5/6. This then triggers inactivation of a cytoplasmic "destruction complex" that limits cytoplasmic β-catenin, permitting its stabilization and nuclear availability. The TCF/Lef family of transcription factors are then able to utilise β-catenin as a transcriptional co-activator, and instigate target gene expression [15]. Amplification of canonical Wnt signalling can be achieved through the participation of another set of receptors, the leucine-rich repeat-containing G-protein coupled receptors (LGR4, 5, 6) and their ligands, the R-Spondins (Rspos) [16]. LGR-Rspo complexes at the cell membrane decrease the endocytic turnover of Frizzled-LRP5/6 by neutralising the ubiquitin ligases RNF43 and ZNRF3 [17]. Oncogenic Wnt/β-catenin signalling is best exemplified in colorectal cancers where activating mutations in β-catenin or loss of APC (Adenomatosis Polyposis Coli) function (a key destruction complex protein) results in high cytoplasmic and/or nuclear β-catenin [18], constitutive β-catenin-TCF/Lef activity and expression of canonical Wnt target genes, such as *MYC* and *CCND1* [15]. In other cancers such as medulloblastoma, however, Wnt pathway activation is associated with a more favourable clinical subtype distinct from the more aggressive c-MYC driven subtype [19].

In neuroblastoma, mutations in Wnt pathway components have only been reported very recently [20], and identified mutations predicted to have high functional impact in a Wnt geneset defined by the KEGG database, and included *NFATC1, FBXW11, TP53, AXIN2, LRP5, CCND1, FZD9, DVL2, FOSL1, WNT7B, VANGL1, LEF1,* and *PPP3CB* genes. Interestingly, the latter three gene mutations introduce premature termination, suggestive of a tumour suppressive role of Wnt signalling in neuroblastoma. Other studies in neuroblastoma have suggested that oncogenic deregulation of Wnt signalling occurs, primarily based on over-expression of canonical Wnt pathway target genes identified in other tissues and cancers. Examples include high *FZD1* expression associated with chemoresistance [21], FZD6 marking highly tumorigenic stem-like cells in mouse and human neuroblastoma [22], and FZD2-dependent proliferation of neuroblastoma lines [23]. Furthermore, deregulated Wnt has been suggested to drive the over-expression of *MYC* in non-*MYCN* amplified (non-MNA) high-risk neuroblastomas [24]. Conversely, however, another study utilising chemical agonists and inhibitors of the Wnt pathway has suggested that Wnt signalling hyperactivation directs neuroblastoma cells to undergo apoptosis, and inhibition of Wnt signalling blocks proliferation and promotes neuroblastoma differentiation [25].

Our previous work reported high expression of the Wnt modulator LGR5 in a subset of neuroblastoma cell-lines as well as poorly differentiated primary neuroblastomas [26]. Using a TCF/Lef reporter assay (TOPFLASH), we showed that three LGR5-expressing neuroblastoma cell-lines with different oncogenic drivers, SK-N-BE(2)-C (MNA), SH-SY5Y (*ALK* mutant) and SK-N-AS (*NRAS* mutant) displayed highly inducible β-catenin-TCF/Lef-regulated transcription when treated with recombinant Wnt3a and R-Spondin 2 (Rspo2), with a strong requirement for LGR5/Rspo2 apparent for maximal induction, as Wnt3a/Rspo2 induction of TOPFLASH was at least 5-fold greater than with Wnt3a alone. Although these neuroblastoma cell lines underwent apoptosis after short-interfering RNA (siRNA)-mediated LGR5 knockdown, depletion of β-catenin did not affect cell survival. This suggested that apoptosis after LGR5 depletion occurred independently of Wnt/β-catenin signalling, and further analyses demonstrated a novel pro-survival regulatory influence of LGR5 on MEK/ERK signalling, independent of Wnt/β-catenin signalling [26]. This dual regulatory capacity of LGRs was subsequently also demonstrated in skin carcinogenesis [27].

Although our previous study showed that several established target genes of canonical Wnt signalling were induced in the neuroblastoma cell lines treated with Wnt3a/Rspo2, including *AXIN2* and *LEF1*, we found that *SOX2*, *MYC* and *MYCN*, which were shown to be Wnt pathway target genes studies in other tissue systems (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/), did not exhibit strong induction. In fact, MYC and MYCN protein levels, as well as the activated kinases Akt and ERK, were reduced after Wnt3a/Rspo2 treatment [26]. This, together with the seemingly discrepant literature on Wnt-mediated effects on neuroblastoma cells, prompted us to identify *bona fide* Wnt target genes in neuroblastoma using RNA sequencing of SK-N-BE(2)-C cells treated with Wnt3a/Rspo2, and thereafter correlate the neuroblastoma Wnt signature with clinical parameters. These analyses, together with our evaluation of Wnt3a/Rspo2 effects on neuroblastoma cell biology, reveal that Wnt regulates recently discovered drivers of differentiation such as *EPAS1* [28] and *BMP4* [29] in cell lines that undergo differentiation. Our study also demonstrates further complex interactions between Wnt signalling and other regulatory pathways likely to be involved in neuroblastoma development.

Materials and Methods

Neuroblastoma Cell Lines and Culture Conditions

Neuroblastoma cell lines were from Carmel McConville (University of Birmingham). The cell lines were grown in DMEM/Nutrient Mixture F-12 Ham (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS) (Life Technologies), 200 mM L-Glutamine (Sigma), 100 units/ml penicillin, 0.1 mg/mL streptomycin (Sigma) and 1% (v/v) non-essential amino acids (Life Technologies). Prior to stimulation with growth factors, cells were grown in the same medium under low serum conditions, containing 1% (v/v) FBS for 24 hours.