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Neuroblastoma Associated Genes Are Enriched in Trunk Neural Crest

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Abstract

Neural crest is a remarkable transient cell population present during embryogenesis, with the ability to differentiate into a multitude of cell types. Depending on anterior to posterior position, neural crest derivatives (cranial, vagal, trunk and sacral, respectively) migrate and commit to restricted lineages. Neuroblastoma is a tumor of infancy and locates along sympathetic ganglia. Based on tumor location and tumor cell gene expression, neuroblastoma is believed to arise from cells of the trunk neural crest-derived sympathetic nervous system. Here we establish the gene expression pattern of trunk enriched- as well as neuroblastoma associated genes over time during early embryogenesis. We show that genes associated with trunk neural crest development as well as neuroblastoma progression are highly expressed at time points when trunk neural crest cells delaminate from the neural tube, undergo epithelial-to-mesenchymal transition and migrate throughout the embryonic body. Using recently established crestosphere cultures – *in vitro* maintenance of multipotent and self-renewing neural crest stem cells – we confirm that diagnostic and prognostic markers of neuroblastoma are highly enriched in trunk- as compared to cranial neural crest. Our data strengthen a trunk neural crest origin for neuroblastoma

Keywords: Neuroblastoma; Neural crest; Trunk neural crest; Crestospheres; Patient-derived xenograft

Introduction

Neuroblastoma is the most common malignancy in infants less than 1 year. Patients can, in rare cases, be born with their tumor, suggesting that priming and/or initiating events occur already during embryogenesis and fetal development [1]. Neuroblastomas are found along sympathetic ganglia with the abdominal region and adrenal glands being the most common sites. Previous studies comparing spatial distribution of common neuroblastoma cell markers with that in healthy human embryos and fetuses have demonstrated that neuroblastoma cells share an expression profile with neural crest-derived sympathoadrenal progenitor- and sympathetic neuronal cells [2-5].

Neural crest is a remarkable multipotent transient cell population during embryogenesis with the ability to form a large variety of different cell types such as chondrocytes, chromaffin cells and melanocytes [6-9]. Neural crest divides into four different derivatives depending on its location along the vertical axis of the embryo [8]. Ordered from anterior to posterior position, these derivatives are designated as cranial, vagal, trunk and sacral neural crest. While the ability to form some cellular lineages (e.g. cells of the peripheral nervous system, melanocytes and smooth muscle) are shared between all neural crest populations, they also give rise to axial level-specific derivatives [6-8]. In order for neural crest cells to migrate and populate the embryonic body, they delaminate from the neural tube to undergo epithelial-to-mesenchymal transition (EMT), a feature shared with cells of many cancer types. Neural crest development has been extensively studied using *in vivo* models in a variety of species [10-13]. The lack of protocols for successful *in vitro* culturing of primary neural crest cells that maintain neural crest identity, multipotency and self-renewing capacity has however halted understanding of mechanisms behind neural crest-associated birth defects and malignancies. Recent reports demonstrate conditions that fulfill all criteria for valuable *in vitro* cultures of cells (denoted crestospheres) from cranial and trunk [14-16] neural crest.

The presence of axial level specific neural crest populations has been known for decades, and whereas designated cell lineage commitment of these populations indeed has been elucidated in detail, improved and novel techniques have raised the question of multiple progenitors for at least some cell types [8]. For example, a recent study using cell lineage tracing and single cell RNA sequencing found that chromaffin cells of the autonomic nervous system not only originate from a common sympathoadrenal precursor cell but also from Schwann cell precursors, i.e., peripheral glia stem cells [17]. The adrenergic medulla is hence composed of neural crest cells as well as Schwann cell precursors, proving that revisiting dogmas of cell type specific origins may be of importance for our understanding of embryonic and tumor development [17].

Neuroblastoma is believed to originate from progenitor cells of the trunk neural crest-derived sympathetic nervous system [18]. Here, we investigate the expression of trunk neural crest enriched markers in cell types of different cancer forms and find that these genes are expressed at high levels in human neuroblastoma and neuroblastoma patient-derived xenograft (PDX) models as compared to cells from other tumor forms. Using the same approach, we show that neuroblastoma associated genes are highly expressed in wild type chick embryos at time points of neural crest cell migration and specification. Furthermore, neuroblastoma associated genes are enriched in crestospheres derived from trunk- as compared to the cranial axial level. Our results validate previous studies suggesting a trunk neural crest origin for childhood tumor form neuroblastoma.

Materials and Methods

Chick embryos

Chick embryos were obtained from commercially purchased fertilized eggs. For trunk crestosphere cultures, stage 13-/14+ (17-

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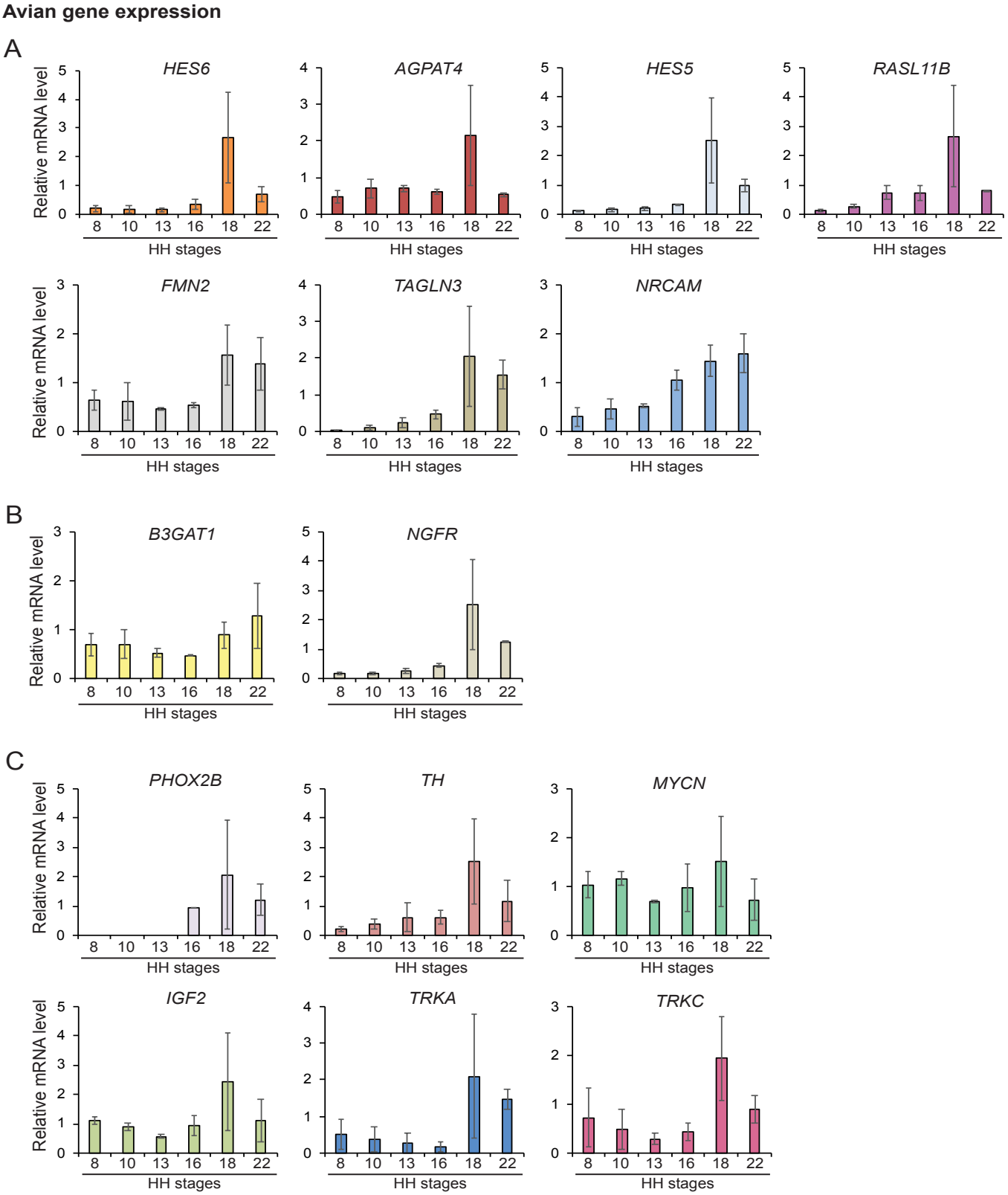

Figure 1: Trunk neural crest enriched genes are expressed at time of neural crest cell migration. Expression of trunk neural crest enriched (A), migrating and late neural crest (B) and neuroblastoma associated (C) genes during embryonic development (HH8 to HH22) in whole wild type embryo as measured by quantitative RT-PCR. Data is presented as mean of n ± SEM (n=2, pool of two to four embryos per biological repeat).

21 somite stage) embryos, and for cranial cultures stage 8-/10 (4-9 somite stage) embryos (staged according to the criteria of Hamburger Hamilton (HH)) were used [19].

Neural tube dissection

Neural tubes from respective axial levels were carefully dissected out from embryos at designated somite stages. For cranial-derived cultures, the very anterior tip was excluded, and the neural tube was dissected until the first somite level as previously described [14]. For trunk-derived cultures, the neural tube was dissected between somite 10-15 as previously described [15,16]. Pools of neural tubes from 4 - 6 embryos were used for each culture.

Crestosphere cell culture

Neural tube explants were cultured in NC medium (DMEM with 4.5g/L glucose (Corning), 7.5% chick embryo extract (MP Biomedicals;

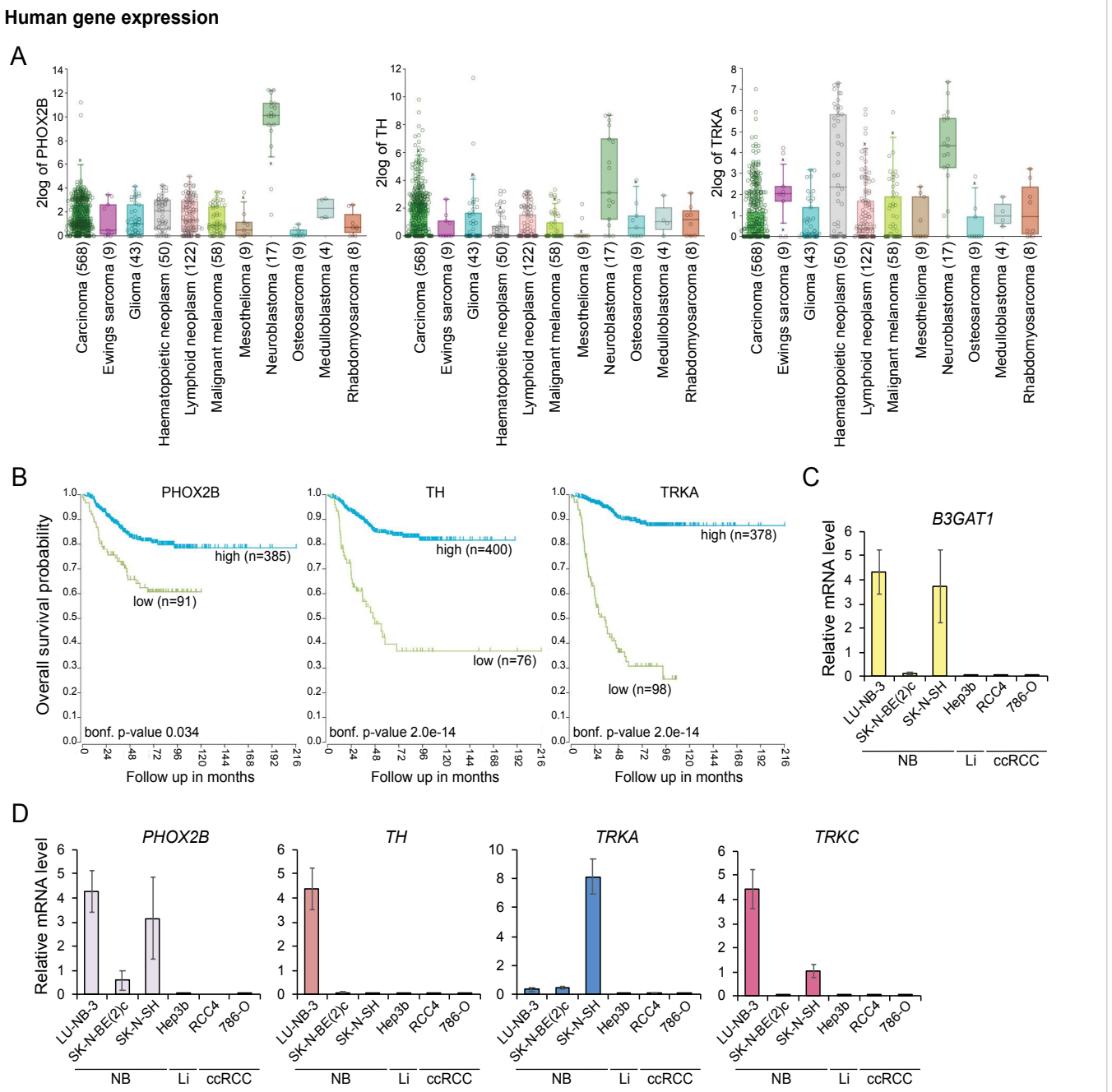

Figure 2: Neural crest and sympathetic neuronal markers are enriched in neuroblastoma. (A) Expression of sympathetic neuronal markers PHOX2B, TH and TRKA in the Cancer Cell Line Encyclopedia (CCLE) data set. (B) Correlation between expression of PHOX2B, TH and TRKA and overall survival in neuroblastoma (data from the KOCAK cohort, 649 tumors). (C-D) Expression of B3GAT1 (C), PHOX2B, TH, TRKA and TRKC (D) in neuroblastoma PDX LU-NB-3 cells, neuroblastoma cell lines SK-N-BE(2)c and SK-N-SH, hepatocellular carcinoma cell line Hep3b and renal cell carcinoma cell lines RCC4 and 786-O. Data is presented as mean of n ± SEM (n=3).

Santa Ana, CA, USA), 1X B27 (Life Technologies; Carlsbad, CA, US), basic fibroblast growth factor (bFGF, 20 ng/ml) (Peprotech; Stockholm, Sweden), insulin growth factor -I (IGF-I, 20 ng/ml) (Sigma Aldrich; Darmstadt, Germany), retinoic acid (RA; 60 nM for cranial and 180 nM for trunk, respectively) (Sigma Aldrich; Darmstadt, Germany), and 25 ng/ml BMP-4 (for trunk) (Peprotech; Stockholm, Sweden)) in low-adherence T25 tissue culture flasks. Due to rapid degradation, retinoic acid and BMP-4 were re-added to the culture medium every 2-3 days.

Cancer cell cultures

Neuroblastoma PDX cell culture LU-NB-3 was maintained in serum-free stem cell promoting medium as previously described [20]. The hepatocellular carcinoma cell line Hep3b (ATCC; Manassas, VA, US) and the neuroblastoma cell lines SK-N-BE(2)c (ATCC; Manassas, VA, US) and SK-N-SH were cultured in MEM while renal cell carcinoma RCC4 and 786-O cell lines were cultured in DMEM, supplemented with 10% fetal bovine serum and 100 units penicillin and 10 µg/mL streptomycin. As part of our laboratory routines, all cells were maintained in culture for no more than 30 continuous passages and regularly screened for mycoplasma. LU-NB-3 and SK-N-BE(2)c cells were authenticated by SNP profiling (Multiplexion, Germany).

RNA extraction and quantitative real-time PCR

Total RNA was extracted using the RNAqueous Micro Kit (Ambion; Waltham, MA, US). Wild type whole embryos were carefully mechanically dissociated before lysis, pooling 2 to 4 embryos for each developmental stage. cDNA synthesis using random primers and qRT-PCR was performed as previously described [21]. Relative mRNA levels were normalized to expression of two (Avian; *18S, 28S*) or three (human; *UBC*, *SDHA*, *YWHAZ*) reference genes using the comparative Ct method [22]. (Refer to Supplementary Table 1 for primer sequences).

Statistical analysis

Two-sided student's unpaired *t* test was used for statistical analyses, **p*<0.05, ***p*<0.01, ****p*<0.001. Publicly available datasets (R2: Microarray analysis and visualization platform (http://r2.amc.nl)) were used to analyze gene expression across cell lines from different cancer types (CCLE) and overall survival (KOCAK).

Results

Trunk neural crest enriched genes are expressed at time of neural crest cell migration

A recent study performed RNA sequencing of a labelled cell population specific to trunk neural crest at developmental stage HH18 in chick embryos, a time point when these cells have delaminated from the neural tube and migrate [23]. We used quantitative PCR (qRT-PCR) to analyze how genes derived from this data set, i.e., genes enriched in

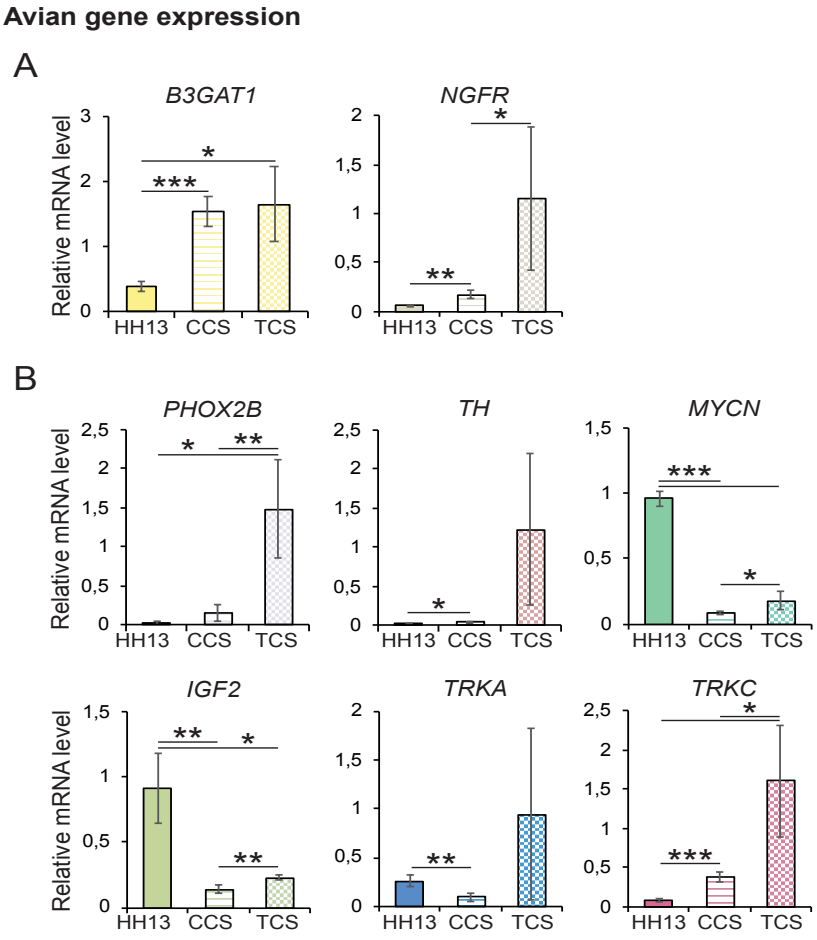

Figure 3: Neuroblastoma associated genes are enriched in trunk neural crest cells. (A-B) Relative mRNA expression levels of neuroblastoma associated genes in HH13 wild type embryo (n=3, pool of at least three embryos per biological repeat), cranial crestospheres (CCS; n=4) and trunk crestospheres (TCS; n=3). Data is presented as mean of n ± SEM, and significance was calculated using two-sided student's t-test; **p*<0.05, ***p*<0.01, ****p*<0.001.

migrating trunk neural crest cells, were expressed during embryonic development within the range of HH8 to HH22 (corresponding to human embryonic week 3 to 5.5, respectively) [23]. In coherence with RNA sequencing data, we found that genes analyzed here (*HES6*, *AGPAT4*, *HES5*, *RASL11B*, *FMN2*, *TAGLN3* and *NRCAM*) were highly expressed around HH18 in whole lysates of wild type chick embryos (Figure 1A).

Diagnostic and prognostic neuroblastoma markers are expressed in neural crest cells

To investigate if also genes associated with neuroblastoma are expressed at time points of trunk neural crest cell migration and specification, we analyzed levels of genes normally expressed in migratory neural crest cells of all derivatives (*B3GAT1*; the gene encoding the enzyme required for formation of the HNK1-epitope, expressed in avian neural crest), later migratory stages and maturing peripheral neurons (*NGFR*) and committed neuroblasts and sympathetic neuronal cells (*TH* and *IGF2*, *MYCN*, *PHOX2B*, *TRKA* and *TRKC*) during the course of embryonic development (HH8 to HH22) [4,24]. Levels of neural crest marker *B3GAT1* varied somewhat inconsistently over time in whole wild type embryo lysates (Figure 1B). In contrast, *NGFR*, a marker of maturing peripheral neurons, was highly expressed at later time points of development (Figure 1B). Markers commonly used in neuroblastoma diagnostics (*PHOX2B*, *TH*, *MYCN*, *TRKA* and *TRKC*), as well as *IGF2* were all expressed during development (Figure 1C). Notably, *PHOX2B* was completely absent at early stages but readily detectable from stage HH16 and onwards, i.e., stages when trunk neural crest cells migrate (Figure 1C). In addition, we measured the expression of these genes particularly in the neural tube portion of the embryo and observed a similar distribution pattern (data not shown).

Trunk neural crest associated genes are enriched in neuroblastoma

To verify the relevance of genes associated with neuroblastoma as well as neural crest and neural crest-derived cell types, we utilized publicly available expression data from the Cancer Cell Line Encyclopedia (CCLE) data set (R2: Microarray analysis and visualization platform (http://r2.amc.nl)). We compared expression among cell lines derived from eleven different tissues with data available from at least four different cell lines in each category respectively (897 cell lines in total). Neural crest migratory gene *B3GAT1* as well as all investigated neuroblastoma associated markers that are also expressed in neural crest-derived sympathetic neuroblasts and neuronal cells (*PHOX2B*, *TH*, *MYCN*, *IGF2*, *TRKA* and *TRKC*) were enriched in neuroblastoma as compared to cell lines from other cancer forms (Figure 2A and Supplementary Figure S1A). As expected, *MYCN* was highly enriched in neuroblastoma as compared to other cell lines, and of note, also *PHOX2B*, *TH* and *TRKA* were more highly expressed in neuroblastoma than in cell lines from any other origin (Figure 2A and Supplementary Figure S1A). Employing the publicly available KOCAK data set of 649 neuroblastomas demonstrated, with the exception of *IGF2*, that low expression of neural crest and sympathetic neuronal markers correlated with poor outcome in this disease (Figure 2B and Supplementary Figure S1B). We confirmed these findings by analyzing gene expression in three neuroblastoma cell cultures (*MYCN* amplified neuroblastoma PDX cell line LU-NB-3, conventional *MYCN* amplified SK-N-BE(2)c and *MYCN* nonamplified SK-N-SH cell lines) as well as in a hepatocellular carcinoma cell line (Hep3b) and two renal cell carcinoma cell lines (RCC4 and 786-O) [20,25]. In coherence with results from the CCLE data set, neuroblastoma associated genes were highly enriched in the neuroblastoma cell cultures as compared to hepatocellular and renal cell carcinoma cell lines (Figures 2C-2D and Supplementary Figure S1C). As expected, *MYCN* was highly expressed in SK-N-BE(2)c and LU-NB-3 MYCN amplified cells and low in SK-N-SH *MYCN* nonamplified cells.

Neuroblastoma associated genes are enriched in trunk neural crest cells

One way to investigate the origin of neuroblastoma has been to compare expression of genes and proteins in neuroblastoma cells with that in healthy non-transformed cells of corresponding tissues [2-5]. Having established that neuroblastoma derives from neural crest cells, we approached the question of neural crest axial level specificity for neuroblastoma progenitor cells by utilizing the recently developed method of crestosphere cultures. We compared gene expression in crestospheres derived from cranial with those from trunk neural crest [14-16]. While there were no differences in expression levels of *B3GAT1*, the late migratory marker *NGFR* was significantly elevated in trunk crestospheres as compared to cranial crestospheres (Figure 3A). Quantification of expression of neuroblastoma associated genes showed that all investigated markers were readily enriched in trunk crestospheres, in particular *PHOX2B*, *TH* and *TRKC* (Figure 3B).

Discussion

Neuroblastoma is a tumor of infancy and with a small subset of patients being born with their tumor, there are reasons to believe that tumor priming or initiating events occur during embryonic and/or fetal development. Earlier studies have shown that neuroblastomas and human sympathetic neuroblasts share a common gene expression profile, but data depicting which precursor cells that give rise to neuroblastoma is absent [5]. We have used chick embryos to isolate cell type specific subpopulations during early time points of embryogenesis. RNA sequencing data recently provided a set of genes enriched in trunk neural crest at developmental stage HH18, a time point when trunk neural crest cells migrate and commit to their various derivative lineages [23]. We confirmed that these genes are expressed during early development and most of them indeed peak around HH18. Neuroblastoma associated markers known to be expressed during normal SNS development could also be detected throughout these early developmental time points with the exception of *PHOX2B* that was expressed only from stage HH16 and onwards, indicating co-expression of trunk enriched- and neuroblastoma associated genes during embryogenesis.

Genes expressed during normal sympathetic neuronal differentiation and in neuroblastoma specimens were highly enriched in neuroblastoma cell lines as compared to cell lines of other neoplastic origin. In line with undifferentiated neuroblastomas being more aggressive, low expression of sympathetic neuronal markers, indicating a more neural crest stem-like state, correlate with poor overall survival in a cohort of 649 tumors [26]. An exception to these data is *IGF2*, expressed in human sympathetic neuroblasts until fetal week 8, where high gene expression correlates with poor outcome. Whether *IGF2* is expressed also earlier than embryonic week 6.5 is not known, but these results might suggest that *IGF2* acts as a marker of more immature neural crest cells [4].

We recently established a novel method to culture trunk neural crest stem cells *in vitro* [15,16]. Termed crestospheres, these cells derive

from microdissected neural tube tissue and maintain their multipotent and self-renewal capacities. We utilized crestospheres derived from cranial and trunk axial levels to compare expression of neuroblastoma associated markers between these two neural crest cell populations [14-16]. Supporting the notion that neuroblastoma derives from trunk neural crest precursor cells, analyzed genes were highly enriched in trunk crestospheres. In coherence with co-expression data presented here, we have previously shown that trunk-enriched markers are expressed at higher levels in trunk crestospheres [26].

Conclusion

In conclusion, trunk enriched- and neuroblastoma associated genes were expressed at time points of trunk neural crest cell migration, and diagnostic and prognostic markers of neuroblastoma were highly enriched in trunk crestospheres as compared to those derived from cranial crest. By utilizing the chick embryo model system, clinical data sets and newly established crestosphere cell cultures, we strengthen a trunk neural crest origin for neuroblastoma.

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Conflicts of Interest

The authors declare no conflict of interest.

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