The Embryonic Trunk Neural Crest Microenvironment Regulates The Plasticity and Invasion of Human Neuroblastoma Via TrkB Signaling

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ABSTRACT

Multipotent, invasive trunk neural crest cells form the vertebrate sympathetic nervous system (SNS), but mistakes in migration and cell differentiation lead to neuroblastoma cancer. TrkB and its ligand, brain-derived neurotrophic (BDNF) direct neural crest cell migration during normal SNS development, however are overexpressed in a majority of high-risk neuroblastomas. In this study, we examined the functional relationship between TrkB expression and aggressive neuroblastoma and the role of the embryonic microenvironment to regulate neuroblastoma cell plasticity and invasion. We interrogated wildtype and TrkB-manipulated human neuroblastoma cell behaviors and gene expression after in vivo transplantation into the chick embryonic trunk neural tube. TrkB expression correlated with neuroblastoma cell invasion, but gain- or loss-of-function promoted or deterred cell aggressiveness, respectively. Intriguingly, transplanted neuroblastoma cells that remained in the dorsal neural tube down-regulated known tumor-initiation and stem cell markers and showed an improved disease outcome as predicted by computational model simulations. These data support TrkB as predictive of neuroblastoma cell aggressiveness and demonstrate the potential of the embryonic trunk neural crest microenvironment to regulate neuroblastoma invasion and plasticity, likely acting at the dorsal trunk neural tube.

INTRODUCTION

A common feature of embryonic development is that cells are born in one place and have to migrate and regulate their differentiation before connecting with a vital target in a different location. One result of this dynamic morphogenetic event is an intricately connected network of the peripheral nervous system that stretches from the pre-vertebral region of the spinal cord (parasympathetic) throughout the vertebral region (sympathetic). In the sympathetic nervous system, nerve signals are carried from the spinal cord to peripheral targets that include the heart, stomach, and adrenal gland and critically function to maintain mammalian homeostasis. The embryonic trunk microenvironment plays an important role to regulate the movement and differentiation of neural crest cells to form the sympathetic nervous system; however signals between the neural crest and microenvironment remain unclear. Failure of proper sympathetic nervous system development may cause neuroblastoma, a solid cancerous tumor that accounts for half of all cancers in infants (Naranjo et al., 2018). Despite efforts to treat high-risk neuroblastomas with aggressive radiation and chemotherapy, there is still a lack of information about specific target genes that regulate neuroblastoma cell plasticity and invasion. Thus, insights into normal sympathetic nervous system formation and studies at the interface between developmental and tumorigenic signaling may help to identify significant genes and signaling pathways that can be explored to more efficiently treat young children.

Sympathetic nervous system development begins with a stereotyped ventral migration of individual neural crest cells that respond to a successive series of cues to form the primary ganglia adjacent to the dorsal aorta (DA; Kasemeier-Kulesa et al., 2005, 2006; 2010, Dyson et al., 2018). Primary ganglia move as an aggregate dorsally in response to signals in the microenvironment and secreted from the pre-ganglionic axons. Strikingly, the formation of the key connection between pre- and post-ganglionic sympathetic neurons requires the secretion of brain-derived

neurotrophic factor (BDNF) from pre-ganglionic axons that is bound by receptor tyrosine kinase B (TrkB) receptors on the developing post-ganglionic sympathetic neurons. Although the transformation from normal sympathetic nervous system development to neuroblastoma is unclear, a strong correlation exists between TrkB expression and poor prognosis (Park et al., 2008; Brodeur et al., 2009). However, the function of TrkB to regulate the plasticity and invasion of human neuroblastoma remains unresolved. Further, given the tight link between sympathetic nervous system formation and neuroblastoma initiation, it is unclear whether signals within the embryonic trunk neural crest microenvironment that regulate normal sympathetic nervous system formation can mitigate neuroblastoma.

We previously developed a chick embryo transplantation model and used this to discover that aberrant regulation of neural crest developmental genes promoted plasticity and invasiveness in neural crest-derived human malignant melanoma (Kulesa et al., 2006; Bailey et al., 2012; Kulesa et al., 2013; Bailey and Kulesa, 2014). Specifically, metastatic melanoma cells exploited neural crest-related receptor tyrosine kinases to increase plasticity and facilitate invasion; primary melanocytes actively suppressed these responses under the same microenvironmental conditions (Bailey et al., 2012). Further, a subset of human metastatic melanoma cells re-expressed melanocyte-like genes after exposure to nerve growth factor (NGF) signals in the chick embryonic trunk microenvironment (Kasemeier-Kulesa et al., 2018a, 2018b). Thus, our chick embryo transplant model offers a unique, accessible method to observe and assess the role of genes to regulate individual human neural crest-derived cancer cell behaviors and compare changes in gene expression of invading versus non-invading transplanted cells to identify signals within the embryonic neural crest microenvironment to regulate tumor cell plasticity and invasion.

Here, we examined the correlation between TrkB expression and human neuroblastoma cell behaviors and the ability of the embryonic trunk neural crest microenvironment to regulate TrkB and other genes involved in cell invasion and plasticity. We refer to the embryonic trunk neural crest microenvironment as the dorsal neural tube and surrounding paraxial mesoderm through which neural crest cells travel. We hypothesize that enhanced expression of TrkB in human neuroblastoma cells leads to aggressive cell behaviors. To test this, we transplanted human neuroblastoma cells with different levels of TrkB expression into the chick trunk neural tube at a developmental stage corresponding to host neural crest cell migration. We quantified neuroblastoma cell invasion patterns after 48hrs of egg reincubation in non-manipulated cells and after alteration of TrkB expression. To determine the influence of the chick embryonic neural crest microenvironment to regulate human neuroblastoma cell plasticity, we examined gene expression profiles of cells prior to transplantation and after cells were isolated from the embryo at 48hrs. Our results elucidate the in vivo function of TrkB and further strengthen our model that signals within the embryonic neural crest microenvironment are capable of promoting, inhibiting and regulating the potential of human neural crest-derived cancer cells.

RESULTS

Human neuroblastoma cells placed in culture are not innately invasive

To examine the baseline migratory ability of human neuroblastoma cells in vitro, we plated wildtype cells (SHSY5Y and LAN5) on fibronectin and poly-lysine coated glass bottom plates and collected time-lapse images to record cell behaviors. We observed cells from both cell lines to display protrusive extensions, random movement, but little net displacement (LAN5 is shown in Fig. 1A,B; Supplemental Movie S1). Interestingly, the SHSY5Y cells preferentially formed neurospheres, a phenotype documented with this cell line (ATCC #CRL-2266). Similarly, when

hanging drops of the cell lines were formed prior to plating in culture, cell clusters remained cohesive with very few cells escaping from a typical cluster (LAN5 is shown in Fig. 1C), supporting evidence for a lack of invasiveness.

Human LAN5 neuroblastoma cells with high TrkB expression show aggressive invasion into the chick embryonic trunk microenvironment including migration into typical neural crest cell free zones

Embryonic trunk neural crest cells follow either a medioventral or dorsolateral migratory pathway depending on the timing of exit from the dorsal neural tube (Dyson et al., 2018). Inhibitory signals expressed within the caudal somite sculpt neural crest cells through the rostral somite halves. Cells that migrate along the medioventral pathway travel to near the dorsal aorta and are sculpted into the primary sympathetic ganglia anlagen (Kasemeier-Kulesa et al., 2005; 2006). Later emerging cells coalesce in a dorsal location to form the sensory ganglia. After pausing in a ventral location adjacent to the dorsal aorta, the primary sympathetic ganglia migrates as a cluster of cells towards the ventral root to synapse with pre-ganglionic axonal projections from the neural tube and wire the circuit of the sympathetic nervous system. We recently discovered that TrkB/BDNF signaling is crucial in the dorsal migration and positioning of the permanent chain of sympathetic ganglia cells (Kasemeier-Kulesa et al., 2015). Mis-regulation of TrkB/BDNF signaling, either by loss of TrkB function or ectopic placement of a BDNF source, or re-positioning of the pre-ganglionic axonal projections led to aberrant migration of the sympathetic ganglia and failure to connect the neural circuit (Kasemeier-Kulesa et al., 2015). Together, this suggested that TrkB regulation may offer important insights into neuroblastoma pathogenesis.

Using the embryonic trunk neural crest migratory pathway framework and insights from normal sympathetic nervous system development, we asked whether human neuroblastoma cells (LAN5, SHSY5Y, NB1643) with a variety of TrkB/BDNF expression (Fig. 1D; high, moderate, low, respectively) transplanted into the chick embryonic trunk neural crest microenvironment (Fig. 1E) invade in an uncontrolled or programmed manner. The latter scenario would suggest that human neuroblastoma cells respond to embryonic signals that typically pattern migrating embryonic trunk neural crest cells. To address this, we transplanted YFP-labeled human LAN5, SHSY5Y, and NB1643 neuroblastoma cells cultured in hanging drops (Fig. 1E, Step1) into the trunk dorsal neural tube of HH10-12 chick embryos, caudal to the last formed pair of somites (Fig. 1E, Step2). After 48 hours of egg re-incubation and embryo harvest, fixation and immunohistochemical staining for migrating neural crest cells (HNK-1) and neurogenesis (tyrosine hydroxylase (TH)), embryos were optically cleared with FRUIT (Hou et al., 2015) and optically sectioned using 3D confocal imaging (Fig. 1E, Step2).

To test our hypothesis that high TrkB expression promotes aggressive neuroblastoma cell behaviors, we transplanted high TrkB expressing LAN5 human neuroblastoma cells (Fig. 1F) into the trunk dorsal neural tube and assessed the cell invasion pattern. Strikingly, LAN5 cells aggressively invaded the chick embryonic microenvironment (Fig. 1F). After 48 hours, cells emigrated from the transplant site in chains or clusters of 2-3 cells, extended long processes typically into neural crest occupied regions (i.e. rostral somite; Fig 1F,G). Cells that remained within the transplant site (non-migratory populations) persisted as a discrete cluster (Fig. 1F,G). Of the cells that emigrated from the dorsal neural tube and invaded the adjacent somitic tissue, ~70% were located in the rostral somite and co-mingled with host neural crest cells (Fig. 1G). When we measure the distance migrated into the embryo, we find a binomial distribution of cells with one cluster between 100-200um from the dorsal neural tube midline and another smaller

subpopulation located approximately 400-500um from the dorsal neural tube midline (Fig. 1G). Analysis of tissue sections of transplanted embryos re-incubated and harvested at 48, 72, and 96 hrs revealed the ventral locations where LAN5 neuroblastoma cells invaded, including preference to the ventral root (VR), dorsal root (DRG) and sympathetic ganglia (SG), and subregion (UDL) between the dorsal neural tube and dorsal root ganglia (Fig. 1H).

Human SHSY5Y neuroblastoma cells with moderate TrkB expression invade the chick embryonic trunk neural crest microenvironment in a programmed manner

We find that SHSY5Y cells invaded the chick embryonic neural crest microenvironment with 5 distinct features. First, invading SHSY5Y neuroblastoma cells did not re-form tumors, but a subset of cells emigrated from the transplantation site (Fig.1I). Second, SHSY5Y cells exited the transplant in a radial pattern and invaded the tissue along the medioventral migratory pathway, similar to the route followed by the first emerging trunk neural crest cells (Fig. 1I). Third, SHSY5Y cells moved along the dorsal neural tube in the anterior and posterior directions (Fig. 1I), similar to host trunk neural crest cell movements observed by Yip and colleagues (Yip et al.,1997). Fourth, migrating SHSY5Y cells extended filipodia to contact neighboring SHSY5Y cells such that the invasion pattern resembled a follow-the-leader pattern of collective cell migration (Fig. 1I). Fifth, SHSY5Y cell invasion tended to be along trajectories through the rostral rather than caudal somite in a pattern that mimicked the host neural crest (Fig. 1J-K). We observed very few SHSY5Y cells that invaded the caudal somite halves (Fig. 1K); SHSY5Y cells that were located within the caudal somite displayed protruding filipodia to contact the rostral somite tissue (Fig. 1I). Invasive SHSY5Y cells reached ventral neural crest target sites by 72 hrs (Fig. 1L) and a significant number of cells were located in the subregion (UDL) between the dorsal neural tube and dorsal root ganglia at 96 hrs after transplantation (Fig. 1L). Thus, human SHSY5Y neuroblastoma cells invaded the chick trunk embryonic neural crest

microenvironment in a controlled manner, preferentially moved through the rostral somite and reached neural crest cell targets.

Human NB143 cells with low TrkB expression remain at the transplant site with very few cells that escape to invade the chick embryonic trunk neural crest microenvironment

We continued with our hypothesis that TrkB expression is a marker of aggressive neuroblastoma cell behavior. Having procured low TrkB expressing NB1643 human neuroblastoma cells, we transplanted cell clusters into the chick trunk dorsal neural tube as described above. Interestingly, NB1643 neuroblastoma cells failed to emigrate from the transplant site and did not invade the surrounding microenvironment (Fig. 1M). We observed very few NB1643 cells (typically <5% of the average cell cluster size) to escape from the transplant site and most cells remained less than 50 um from the transplant (Fig. 1M,N). Escaped NB1643 neuroblastoma cells appeared as individuals and did not move as a collective group of cells; few cells moved through the rostral versus caudal somite (Fig. 1O) and reached the dorsal root ganglia (Fig. 1P) by 96 hrs. NB1643 cells that remained in the transplanted cluster were tightly adhered to each other with very few cellular protrusions poking out from cells on the periphery (Fig. 1M). Thus, the low expression of TrkB in 1643 human neuroblastoma cells correlated with the significant loss of aggressive behavior related to invasion with very few cells escaping to reach dorsally-located neural crest cell targets.

Non-neuroblastoma control CHLA-10 cells fail to invade the chick embryonic trunk neural crest microenvironment

Thusfar, we have observed that human sympathetic neuron-derived cancer cells (SHSY5Y, LAN5, NB1643) invade the chick embryonic trunk neural crest microenvironment with

aggressiveness that correlates with TrkB expression. To determine whether non-sympathetic neuron derived cancer cancer cells invade the embryonic trunk neural crest microenvironment, we transplanted clusters of CHLA-10. CHLA-10 cells are derived from a primitive neuroectodermal tumor (PNET) that normally manifest in the central nervous system. Expression analysis of CHLA-10 cells revealed TrkB RNA levels to be roughly equivalent to SHSY5Y cells (Fig. 1E). Therefore, comparison of cells expressing TrkB but from a different origin would determine whether additional factors other than TrkB contribute to the cell's ability to migrate through the embryo. When transplanted into the chick embryonic trunk neural crest microenvironment, CHLA-10 cells failed to invade the embryo and remained as a cohesive clump at the transplant site (Supplemental Fig. S1A).

Knockdown of TrkB in LAN5 and SHSY5Y human neuroblastoma cells limited the ability of cells to invade the chick embryonic trunk neural crest microenvironment

To further examine the role of TrkB on the invasive ability of human neuroblastoma cells in the chick embryonic trunk neural crest microenvironment, we used shRNA to knockdown TrkB on LAN5 human neuroblastoma cells (Fig. 2A-C). We achieved a 72% knockdown at the protein level (compared to wild-type cells, Supplemental Fig. S1B). When these cells were transplanted into the chick embryonic trunk neural crest microenvironment, we discovered a drastic reduction in the number of cells that exited from the transplant site (Fig. 2A,B). That is, ~100 cells invaded per embryo in WT:LAN5 transplants, versus ~2 cells per embryo in LAN5:shTrkB transplants (Fig. 2C). Therefore, TrkB knockdown in highly invasive human neuroblastoma cells limited the ability of the cells to readout and respond to potential signals in the chick embryonic trunk neural crest microenvironment to move aggressively into the surrounding tissue.

In comparison, when we knocked down TrkB on the moderate TrkB expressing cell line, SHSY5Y, we find a significant reduction in the number of cells that emigrated from the transplant site (Fig. 2D-F). The majority of cells in a typical transplant remained at the transplant site (Fig. 2D,E). We observed only small groups of cells (<5) a short distance away from the transplant (within 50um); very few individual cells emigrated further but were less than 150um away. Comparing cell invasion characteristics between manipulated versus non-manipulated cells, we find only approximately 14 out of 300-400 cells per embryo invade (SHSY5Y:shTrkB) versus a five-fold higher 74 cells per embryo in WT:SHSY5Y cell transplants (Fig. 2F). Lastly, there were slightly more cells emigrating from the transplant site in SHSY5Y:TrkB knockdown compared with LAN5:TrkB knockdown cells (compare Fig. 2C with Fig. 2F).

Overexpression of TrkB in human NB1643 cells increased cell invasiveness in the chick embryonic trunk neural crest microenvironment

Human NB1643 cells have low TrkB expression and were observed to be poorly invasive when placed into the chick embryonic trunk neural crest microenvironment (Fig. 1M). We therefore asked whether NB1643 cells would become aggressive if TrkB expression was enhanced. When we over-expressed TrkB on NB1643 cells (FLTrkB-NB1643; Supplemental Fig. S1C) and transplanted cells into the chick embryonic trunk neural crest microenvironment, we observed a dramatic increase in cell invasion (Fig. 2G-I). Individual and groups of cells extended from the transplant site onto the host trunk neural crest cell migratory pathways (Fig. 2G,H). Cells displayed more protrusions than their wild-type counterparts, but not as significant an increase compared with wildtype SHSY5Y or LAN5 cell lines (Fig. 2H). Cells were observed in chain-like arrays and also as individual cells after exiting the transplant site (Fig. 2H). Analyzing the number of cells that invade the embryo per transplant, we find that approximately 3 cells per

embryo invade in WT:NB1643 transplants, but over 50 cells per embryo invade in

NB1643:FLTrkB transplants, a greater than 10-fold increase (Fig. 2I).

(We are evaluating whether to include the next 3 paragraphs)

Human SHSY5Y-FL TrkB neuroblastoma cells exit the dorsal neural tube transplant site in significantly higher cell numbers and reach host neural crest cell peripheral targets faster than SHSY5Y-WT, LAN5-WT, and LAN5-shD TrkB cells

To more accurately determine the dorsoventral cell positions to which transplanted human neuroblastoma cells traveled, we analyzed thin cryosections reconstructed in 3D to count the number of cells within the transplant site and peripheral locations at 48, 72, and 96 hrs after transplantation (Fig. 2J; Graphs in progress?). We discovered distinct spatio-temporal patterns of the human neuroblastoma cell transplantations when we compared the 2 wildtype (SHSY5Y and LAN5) and TrkB manipulated (SHSY5Y-FL TrkB, LAN5-shD TrkB) cells (Fig. 2J). First, SHSY5Y and SHSY5Y-FL TrkB neuroblastoma cells showed significantly more cells exited from the transplant site and into the host chick embryonic trunk neural crest microenvironment than either LAN5-WT or LAN5-shD TrkB (Fig. 2J). By 96 hrs post-transplantation, SHSY5Y neuroblastoma cells showed the highest percentage of cells that had exited the dorsal neural tube transplant site. In contrast, LAN5-shD Trk had the highest percentage of cells that remained at the transplant site (Fig. 2J). Furthermore, SHSY5Y-FL TrkB neuroblastoma cells showed the largest number of cells in host non-neural crest locations (Fig. 2J). These locations included spaces between the dorsolateral and ventral migratory pathways and the typically neural crest cell free perinotochordal region (Fig. 2J). Lastly, we observed cell debris and unhealthy LAN5-WT and LAN5-shD TrkB in the embryonic microenvironment at 72 hrs and 96 hrs after transplantation (Fig. 2J).

A comparison of the timing to reach host neural crest target sites (dorsal root ganglia (DRG), sympathetic ganglia (SG), and dorsolateral pathway) showed that SHSY5Y neuroblastoma cells reached host neural crest cell peripheral locations faster than the LAN5 cells (Fig. 2K). Within 48 hrs of transplantation, SHSY5Y-FL TrkB cells reached the dorsal root and sympathetic ganglia (Fig. 2K). SHSY5Y-wildtype cells reached the sympathetic ganglia 24 hrs later (Fig. 2K). In contrast, LAN5-WT neuroblastoma cells reached the dorsal root ganglia between 48-96 hrs, but LAN5-shD TrkB cells never reached the ventrally located sympathetic ganglia (Fig. 2K). Lastly, SHSY5Y-FL TrkB neuroblastoma cells were found along the dorsolateral migratory pathway, typical of host neural crest cells that become melanocytes in the skin, a day earlier than SHSY5Y-WT and LAN5-WT cells (Fig. 2K). LAN5-shD TrkB neuroblastoma cells did not travel along the dorsolateral migratory pathway (Fig. 2K). Together, these data demonstrate that TrkB expression correlated with the number of invading human neuroblastoma cells and the timing at which cells arrived at host neural crest target sites.

Neural crest cell ablation did not affect neuroblastoma cell invasion of the chick embryonic trunk microenvironment

To assess whether invasive transplanted human neuroblastoma cells were simply using migrating neural crest cells as a scaffold, we ablated host premigratory neural crest cells immediately before transplantation of LAN5 neuroblastoma cells (Fig. 2; see data from Jen on M/Jen/neuroblastoma/ablations and transplantations, 10-25-17). We find that LAN5 neuroblastoma cells traveled along host neural crest cell migratory pathways and showed a higher percentage of cells invading the embryo in ablated embryos (Fig. 2). This suggests that human neuroblastoma cells were able to navigate the embryo neural crest cell migratory pathways without the host neural crest cells.

Transplanted human neuroblastoma cells show enrichment for differentiation, neurogenesis and development genes and down-regulate known tumor-initiating and stem cell genes after exposure to the chick embryonic trunk neural crest microenvironment

Neural crest-derived metastatic melanoma cells transplanted into the chick embryonic head neural crest microenvironment hijack inherent developmental signaling pathways to enhance their metastatic potential, including significant changes in ephrin expression (Bailey et al., 2012; Bailey and Kulesa, 2014). This led us to discover that EphB6 re-expression resulted in a significant loss of transplanted cell intravastion, suggesting its potential as a tumor suppressor (Bailey and Kulesa, 2014). Since our results thusfar show that TrkB expression modulation alters cell behaviors in the chick trunk neural crest microenvironment, it made logical sense to ask whether changes in gene expression were indicative of cell behavioral changes.

To address this, we transplanted human neuroblastoma cells into the embryo as above, and after 48 hours used FACs to isolate the neuroblastoma cells and compare the gene expression profiles between SHSY5Y, LAN5 and NB1643 wildtype cells lines from culture and embryo transplants. Initial heat map clustering classification of the wild type cell lines from culture showed LAN5 and NB1643 are more closely related than SHSY5Y to either cell line, possibly in part due to SHSY5Y as an established, commercially available cell line, whereas LAN5 and NB1643 cell lines were obtained from Children's Oncology Group (COG) and with a potential less time in established culture (Supplemental Fig. S1D). We next profiled human neuroblastoma cells after transplantation into the embryo and exposure to the chick embryonic trunk microenvironment for 48 hours. Correlation analysis of the three cell lines (SHSY5Y, LAN5, NB1643) from culture and after embryonic exposure, showed the three wild type cell lines had high correlation with one another and the three cell lines from embryonic exposure

had moderate correlation with each other (Fig. 3A). When we compared the genes changed after exposure to the embryonic microenvironment from each cell line, we find LAN5 (4,046 genes), SHSY5Y (8,103 genes) and NB1643 (8,776 genes) had large numbers of genes that were significantly reduced or enhanced. When we analyzed genes in common across these three gene sets, we find a surprising number of 1,857 genes in common (Fig. 3B). Of these 1,857 genes, gene set enrichment analysis (GSEA) showed Go Term Enrichment for the genes in common, with the top Go Term classification as Neurogenesis (211 genes out of 1402 in the group) and from the top 15 Go Terms, 47% percent were related to differentiation, neurogenesis or development (Fig. 3C).

Tumor initiating cells and stem cell plasticity are characteristic of aggressive tumors. Gene expression analyses have identified specific markers for tumor initiating (EPAS1, MTOR, VIM, ALDH1 and NOTCH1) and stem (PAX6, RUNX1, ID2, SOX9, ASCL1, MSI1, FGFR2, SOX2, GATA2, NOTCH2, FGFR4 and NES) cells (Fig. 4). When we analyzed RNAseq data from human neuroblastoma cells exposed to the chick embryonic trunk neural crest microenvironment for tumor initiating and stem cell markers, strikingly we found all 5 tumor initiating cell makers were down-regulated in the three transplanted cell lines as compared to cultured cells (Fig. 4A-C). Furthermore, 8 out of 10 (LAN5 and SHSY5Y) and 10 out of 12 (NB1643) stem cell markers were down-regulated as compared to cultured cells (Fig. 4D-F). Together, this showed that signals within the chick embryonic trunk neural crest microenvironment have the potential to regulate the metastatic phenotype of neuroblastoma.

Single cell RNAseq reveals subpopulations do not exist in cultured neuroblastoma cells

To address whether the non-migratory and invasive subpopulations of human neuroblastoma cells we observed after transplantation into the embryo pre-exist within the wildtype subpopulations in culture, we performed scRNAseq profiling of both the bulk population of cultured cells and cells from hanging drop culture (immediately before transplantation into the embryo; Fig. 4G-in progress). When we analyzed 12,367 single cells from cultured human SHSY5Y neuroblastoma cells, and used the Loupe cell browser from 10x Genomics to cluster the cells based on differential gene expression, we found only one gene (HSP90AA1) to be statistically different (Fig. 4G). Profiling of human LAN5 neuroblastoma cells showed similar results (with 4,736 cells) although we did find slightly higher heterogeneity with a handful genes that were differentially expressed across the population (Fig. 4G). Further, when we analyzed 5,062 cells from LAN5 hanging drop culture, there was slight clustering of different groups, but with small numbers of cells with differential expression per group (2-24 genes) and no enrichment for neurogenesis related genes (Fig. 4G?). These results demonstrate that distinct non-migratory and invasive subpopulations of human neuroblastoma cells occur as a result of exposure to the chick embryonic trunk neural crest microenvironment and not resident in three wildtype cultured cell lines.

RNAseq reveals gene expression differences in non-migratory and invasive cells after exposure to the embryonic trunk neural crest microenvironment

Upon determining 2 main behaviors of neuroblastoma cells when transplanted into the neural crest microenvironment, non-migratory (remained at the transplant site) and cells that exited from the transplant and were invasive, we asked whether gene expression profiles were different among these 2 populations. Due to these observations we manually excised the

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cluster of tumor cells that remained in the neural tube, and used FACs analysis to separately harvest the cells that had invaded the embryo, and analyzed each population by RNAseq. Correlation matrix analysis again showed the 3 wildtype cell lines had high correlation with each other. In all 3 cell lines, there was a slight decrease in correlation between the invasive and non-migratory populations, but interestingly, these both had decreased correlation when compared to all wild type cell lines. The invasive cells had the lowest correlation to any wildtype cell line of all comparisons made (Fig. 5A).

We next determined the genes that were significantly altered after embryonic exposure from the non-migratory populations compared to culture. We discovered the set of genes in common across all 3 cells lines to yield a list of 328 genes (Fig. 5B). GO Term enrichment analysis showed that this set of 328 genes categorized into cell adhesion and development (Fig. 5C). Performing the same process for the invasive cell populations, we found 1,677 genes that were altered after embryonic exposure in all 3 cell lines (Fig. 5B). Go Term analysis showed an enrichment for neurogenesis, differentiation, guidance and morphogenesis involved in migration (Fig. 5D). We further analyzed the Go Term alignment and determined the percent of annotations that were changed in either the non-migrated or invasive populations and compared this to random signatures. Interestingly, we found that transplanted neuroblastoma cells that remained within the dorsal neural tube had the highest percent expression of differentiation and cell adhesion genes as compared to random gene signatures genes (Fig. 5E). In contrast, the neuroblastoma cell population had high expression of genes associated with cell proliferation, cell motility and growth. These results indicate a striking difference between non-migrated and invasive cell behaviors and support the significant influence of the embryonic trunk neural crest microenvironment.

Human neuroblastoma cells that remained at the chick dorsal neural tube transplant site showed improved disease outcome as predicted by our computational model

After identifying two different phenotypic and genotypic subpopulations arise after exposure of human neuroblastoma cells to the chick embryonic trunk neural crest microenvironment, we used our recently developed algorithm (Kasemeier-Kulesa et al., 2018) to predict the disease outcome of each subpopulation (Fig. 6). Our model algorithm of neuroblastoma uses a 6-gene input, based on receptor tyrosine kinase signaling, to predict disease outcome and is more accurate for early stage disease than any current clinical prognostic (Kasemeier-Kulesa et al., 2018). Strikingly, in all three cell lines (LAN5, SHSY5Y, NB1643), subpopulations exposed to the embryo produced different predicted outcomes than the corresponding wildtype cell line placed in culture (Fig. 6). For example, using the input of a 6-gene signature obtained from the RNAseq data, our logic model predicted that invasive cell lines (LAN5 and SHSY5Y) have a poor outcome based on the molecular signature after invasion in the embryo. However, the model predicted a better disease outcome of the non-invasive cells that remained at the original transplant site (LAN5=intermediate and SHSY5Y=good outcome) (Fig. 6). Specifically, the algorithm predicted decreases in cell differentiation and apoptosis in the LAN5 invasive cell population; confirmed by the RNAseq GoTerm Analysis (Fig. 5). Similar results showed the SHSY5Y invasive population with upregulated angiogenesis and cell proliferation after invasion into the embryonic trunk neural crest microenvironment.

In contrast, NB1643 transplanted cells that produced very few cells exiting the transplant site (Fig. 2) were now predicted to reach an intermediate to good disease outcome for both the non-migrated and invasive population (Fig. 6). Interestingly, the model predicted that transplanted human NB1643 cells decreased angiogenesis and cell proliferation, eluding to their non-invasive phenotype (Fig. 6). Thus, our model algorithm predicted a favorable disease outcome

for human neuroblastoma cells that remained at the transplant site (after exposure to the chick embryonic trunk neural crest microenvironment) and accurately predicted a poor outcome for the three invasive and cultured cell lines.

DISCUSSION

TrkB/BDNF signaling is critical to trunk neural crest cell migration during normal sympathetic nervous system development (Kasemeier-Kulesa et al., 2015), but the correlation of high TrkB expression with poor prognosis for neuroblastoma cancer in children has led to several unanswered questions. In this study, we examined the function of TrkB during human neuroblastoma cell invasion and ability of the embryonic trunk neural crest microenvironment to mitigate neuroblastoma cell invasion and plasticity. We learned that manipulation of TrkB expression either by gain- or loss-of-function promoted or deterred human neuroblastoma cell invasion after transplantation into the embryonic trunk neural crest microenvironment, respectively. Human neuroblastoma cells that were non-invasive (remained in the dorsal neural tube transplant site) down-regulated known tumor-initiation and stem cell markers. Model simulations then predicted a better prognosis for these non-invasive human neuroblastoma cells. These findings demonstrate that modulation of TrkB signaling controls human neuroblastoma cell invasiveness and suggest a rich source of signals within the embryonic trunk neural crest microenvironment both within the dorsal neural tube and paraxial mesoderm that regulate neuroblastoma cell plasticity and invasion.

The level of TrkB expression correlated with human neuroblastoma cell invasion into the embryonic trunk neural crest microenvironment, strengthening the theme that TrkB signaling is a critical indicator of neuroblastoma invasiveness and potential therapeutic target. Human

SHSY5Y (moderate TrkB expression) and LAN5 (high TrkB expression) showed aggressive invasion into the embryonic trunk neural crest microenvironment, but knockdown of TrkB expression severely limited the invasive ability of these cells in the same in vivo model. That is, a large majority of the transplanted SHSY5Yand LAN5 TrkB knockdown cells remained at the transplant site (Fig. 2A-F). **PUT IN THE WORK OF BRODEUR ON TARGETING TRK signaling but show that its effective but some patients not, and that they were still able to signal through TrkB.** Thus, future analysis of the human neuroblastoma cells that escaped knockdown of TrkB or maintained invasion into the embryonic trunk neural crest microenvironment may help to answer this question.

The avian embryo has emerged as a powerful model organism to examine the invasion and plasticity of human neural crest-derived cancer cells (Kulesa et al., 2006; Hendrix et al., 2007) and more recently the underlying embryonic signals that drive cancer cells to a less aggressive state (Kulesa et al., 2013; Kasemeier-Kulesa et al., 2018). This is supported by work from Castellani and colleagues (2017) who further validated the chick embryo as a source of discovery for signals that regulate cancer cell behaviors. The authors injected a loose assortment of human neuroblastoma cells into the chick embryo trunk between the neural tube and epidermis and reported invasion of cells into the periphery, showing that SEMA3C expression levels correlated with either growth or cell detachment and invasion (Delloye-Bourgeois et al., 2017). Our results here indicate that the transplantation of an aggregate rather than injection of a loose assortment of human neuroblastoma cells into the chick embryonic neural crest microenvironment allows for the identification of highly aggressive cells among the tumor population as a whole (Fig. 1E,I,M). That is, highly aggressive neuroblastoma cells may not be evident when analyzed in bulk or even single cells from culture and support our hypothesis that tumor initiating cells may be present within a larger cell population and only

display cell behavior and gene expression differences when presented with the proper

microenvironment.

Non-neuroblastoma CHLA-10 cells failed to invade the chick embryonic trunk neural crest

microenvironment, but have high expression of TrkB in culture, suggesting non-neural crest-

derived cells may not invade the chick embryonic neural crest microenvironment. In support of

this, we previously showed that human metastatic melanoma cells follow host cranial neural

crest cell migratory pathways after transplantation into the chick embryonic neural crest

microenvironment and revive an embryonic migration program (Bailey et al., 2012). However,

poorly invasive human melanoma cells or human melanocytes tend to remain at the transplant

site (Bailey et al., 2012). Alternatively, the expression of TrkB may be linked to the presence of

BDNF, since TrkB/BDNF is a signaling axis that directs trunk neural crest cells to a final location

to form the sympathetic nervous system (Kasemeier-Kulesa et al., 2015). BDNF levels varied

across the three human neuroblastoma cell lines we analyzed; LAN5 and SHSY5Y had high

TrkB and low BDNF expression relative to each other and NB1643 cells had high BDNF and low

TrkB, relative to each other. Future studies that alter BDNF levels with and without altering TrkB

levels in human neuroblastoma cells and the analysis of subsequent in vivo cell behaviors

should help to address this question.

Human neuroblastoma cells that remained at the transplant site showed enrichment for

differentiation, neurogenesis, and development genes such as EYA1, RUNX1, RARB, and TH.

Further, non-invasive cells showed down-regulation of well-known tumor initiating and stem cell

markers, including CD133, EDNRB, NOTCH1 and MTOR. Together, this was not a complete

surprise. We previously showed that a subset of human C8161 metastatic melanoma cells re-

express a melanocyte-like marker, MART-1 after invasion into the chick embryonic neural crest

microenvironment (Kulesa et al., 2006). By closely matching melanoma cell positions and re-expression of MART-1, and performing co-cultures of C8161 melanoma cells with various chick embryonic tissues along the trunk neural crest cell migratory pathway, we learned that chick day 3.5 dorsal root ganglia was the source signal that drove the re-expression of MART-1 in cells. We went on to show that nerve growth factor (NGF) typically present in the dorsal root ganglia and acts as a neurotrophic factor, reprograms human metastatic melanoma cells to a less aggressive neural crest-like phenotype (Kasemeier et al., 2018). Thus, powerful signals in the embryonic neural crest microenvironment may offer a fertile ground to examine factors that regulate neuroblastoma cell plasticity and invasion.

Changes in gene expression measured after exposure of human neuroblastoma cells to the embryonic trunk neural crest microenvironment suggested that non-invasive cells are less de-differentiated. To test whether this would result in a more favorable neuroblastoma disease outcome, we took advantage of our computational model (Kasemeier-Kulesa et al., 2018) that simulates the network dynamics of 6 developmental genes to determine the output of four cell states that predict disease outcome. Based on the input of a 6-gene input signature obtained from the RNAseq data, our model predicted that LAN5 and SHSY5Y neuroblastoma cells that invaded the embryonic trunk neural crest microenvironment had a poor disease outcome. Specifically, the model algorithm predicted decreased differentiation and apoptosis in the LAN5 invasive cell population; confirmed by the RNAseq GoTerm Analysis (Fig. 3C), with similar results for the SHSY5Y invasive population with upregulated angiogenesis and proliferation (Fig. 6). However, LAN5 and SHSY5Y neuroblastoma cells that remained at the transplant site had changes in their 6 gene signature that led to a more favorable disease outcome prediction (Fig. 6). Thus, the coupling of an in vivo transplant system and computational predictive model of neuroblastoma that is personalized for

individual patient data offer a powerful and novel set of tools to complement current neuroblastoma prognostic indicators and further allow for targeted therapy and response to treatment evaluations.

In summary, the focus of this study was to better understand the function of TrkB signaling and the role of the embryonic trunk neural crest microenvironment to regulate neuroblastoma cell invasion and plasticity. Our data support a model whereby the embryonic trunk neural crest microenvironment sorts out the neuroblastoma tumor initiating population, self-identifying targetable cells. Alternatively, signals within the embryonic neural crest microenvironment may force differentiation of the neuroblastoma cells to drive cells to be less aggressive. Further investigation into signals from the embryonic dorsal neural tube will help to determine how this unique microenvironment is able to control the metastatic behavior of aggressive human neuroblastoma.

MATERIALS AND METHODS

Chick embryos

Fertile white leghorn chick eggs were acquired from a supplier (Centurion Poultry; Lexington, GA) and incubated at 38 degrees to stages HH 10-12 of development (HH; Hamburger and Hamilton, 1951). At this stage 3 ml of albumin was removed from each egg and a window was cut in the shell to visualize the embryo. A solution of 10% India ink diluted with Howard ringer's solution was injected under the blastodisc to visualize the embryo.

Human cell lines and cell culture

Human neuroblastoma cell lines SK-N-BE (2) and NB-1643 were obtained from the Children's Oncology Group Cell Culture and Xenograft Repository (Lubbock, TX). The SHSY5Y cell line was obtained from the American Type Culture Collection (Manassas, VA). SK-N-BE (2) were cultured in RPMI (VWR #45000-396) + 10% FBS, NB-1643 were cultured in Iscove's modified dulbeccos medium (Sigma I3390-500ml) + 20% FBS + 2x Glx (Thermofisher #35050061) + 1x ITS (Sigma I3146-5ml). SHSY5Y were cultured in 1:1 MEM (Corning #10-022-CV): F-12 (Thermofisher #11765054) +10% FBS (or per data-sheets). Cells were maintained at 37 degrees with 5% CO_2 and tested for mycoplasma contamination using the Universal mycoplasma detection kit (ATCC #30-1012K). All cell lines were fluorescently labeled by infecting cultures with pLENTI GAP43: YFP lentivirus. The pLENTI GAP43: YFP construct was created from pGAP43: YFP and pLENTI GAP43: eGFP using standard subcloning techniques.

Neuroblastoma cell transplantation into the chick neural tube

Cells were prepared in hanging drops with Matrigel (BD Biosciences 354234, San Jose CA), at a concentration of 4×10^6 cells/ ml with 0.5% Matrigel for the SHSY5Y cell line, a concentration of 8 x 10^6 cells / ml with 0.5% Matrigel for the NB-1643 cell line and at 8 x 10^6 cells / ml with 1% Matrigel for the SK-N-BE (2) cell line. The hanging drops were prepared 40-48 hours in advance of use and cut using a pulled glass needle to the size of 1 chick somite (approximately 150 um square; 300-500 cells) for transplantation. Using a P-2 pipette, the transplant was placed near the chick embryo and manipulated using a tungsten needle into the dorsal neural tube of the embryo behind the last formed pair of somites. Embryos were left for 1 hour at room temperature to settle before being re-incubated for an additional 48 hours. Afterwards, embryos were harvested, fixed in 4% PFA (from **where)** and washed in PBS.

Cell labeling and 3D Confocal Microscopy

To visualize the host neural crest and neural crest peripheral targets embryos were stained for HNK-1, Tyrosine Hydroxylase(Novus biologicals- NB300-109 , Littleton, CO), and DAPI. Embryos were optically cleared with FRUIT (Hou et al., 2015) to allow for deep tissue imaging of the migrated cells. To clear embryos were incubated in a solution of 8M Urea with 0.1% α-thioglycerol containing increasing concentrations of fructose from 40% – 100%, until embryos equilibrated. Embryos were mounted between coverslips (VWR Micro cover glass No. 1.5 22x 60mm), using a thin layer of vacuum grease (Dow Corning 976V high vacuum grease), in 100% FRUIT solution and were imaged within a week of being cleared. Embryos were mounted with either the dorsal neural tube facing upwards or with the sagittal side of the embryo exposed on the coverslips. Images were collected on a Zeiss 800 or a Zeiss 710 microscope (Zeiss MicroImaging; Thornwood, NY), with a Plan- Apochromat 10X/0.45 M27 objective was typical. Images were at a Z-depth of between 110-300 um, (40-110 slices) , images were taken on two tracks, to get the best signal from staining.

Image analysis

Images were loaded into Imaris x64 (Bitplane; Zurich, Switzerland) where background was subtracted, then cells and the neural crest stream were manually identified using spot detection. A surface was manually built on the body of the transplant after which a distance transformation between the cells and the surface was performed. The distances obtained were loaded into OriginPro (OriginLab Cooperation; Northampton, MA) and plotted in a histogram to visualize the differences in the three migration patterns. Using the position of the neural crest and neuroblastoma cells from spot detection the cells were scored as being in the rostral or caudal somite.

LAN5 WT, LAN5 shD (knockdown of TrkB), SHSY5Y WT, and SHSY5Y FL TrkB

(overexpression of TrkB) cell lines were brought to concentrations of 4 million cells/mL. 1%

Matrigel (BD Biosciences, 354234) was added to the LAN5 cell lines, and 0.5% Matrigel was

added to the SHSY5Y cell lines before they were suspended as 25 uL hanging drops. After 48

hours, these hanging drops added to PBS in a petri dish with a transfer pipette and cut into balls

the size of a somite (approximately 150 um in diameter) using a pulled glass needle that has

been trimmed to functional size. Chicken eggs from Centurion Poultry were incubated for 48

hours on their sides to stage 8-12 somites, which were then prepped for transplant by removing

5 mL of the albumin using a 10 mL syringe, cutting an ovular window in the egg, and injecting a

solution of 1% India Ink (source) in a Ringer solution containing 10% Penicillin Streptomycin

antibiotic under the embryo using a 1 mL syringe. A small tear was made just dorsal to the

transplantation site using the tip of a syringe, and the cell clump was placed into the egg in 4-5

uL of PBS using a p20 pipette with a 20 uL tip. This cell clump was then manipulated into the

neural tube just under the developing somites by a pulled glass needle. After transplantation, a

few drops of the same solution of 10% Penicillin Streptomycin antibiotic in Ringer solution used

to prepare the India Ink solution was added to the egg with a transfer pipette to maintain

moisture and prevent infection, and clear tape was used to seal the window in the egg. The

eggs were then taped closed and allowed to sit at room temperature from anywhere between 15

to 30 minutes before being placed back in the incubator for harvest at 48 hours, 72 hours, and

96 hours post-transplantation.

After harvest, the embryos were placed in 4% PFA for 2 hours at room temperature and then

cleaned with forceps by removing the membranes and developing heart region. Using a

fluorescent stereoscopic microscope, the region of the transplant was located, excised, and

then cryostat sectioned at 12 um slices. After sectioning, the slides were stained with HNK-1

primary antibody (source, 1:25) and Alexa-Fluor 633 goat anti-mouse IgM secondary antibody (Invitrogen, A21046, 1:500) by washing 4x10 minutes with PBSTT (PBS with 0.1% Triton-X), blocking one hour in 10 % goat serum in PBSTT, staining one hour at room temperature in primary antibody in the same blocking solution, washing 4x10 minutes with PBSTT, blocking one hour with the blocking solution, staining for one hour at room temperature in secondary antibody, washing 4x10 minutes in 1:3 diluted block in PBSTT, washing 2x5 minutes in PBS, and mounting with Prolong Gold antifade reagent (Invitrogen, P36930) and 24x50 mm coverslips.

The slides were then imaged using a 633 channel to capture the HNK-1 signal in conjunction with spectral imaging to capture the transplant cells, which heterogeneously expressed their fluorescent tags and were poorly-bound to anti-GFP and anti-mCherry primary antibodies. After imaging, the transplant cells were counted and analyzed based on their position according to seven different locations: the neural tube lumen (the site of transplantation), the neural tube, the upper dorsal lateral pathway, the dorsal root ganglion, the ventral root, the sympathetic ganglion, and ectopic sites that are not characteristic of neural crest cell migration.

LAN5, NB1643 & SHSY5Y in culture bulk RNA-seq

mRNAseq libraries were generated from 500ng of high-quality total RNA, as assessed using the Bioanalyzer (Agilent). Libraries were made according to the manufacturer's directions for the TruSeq Stranded mRNA LT Sample Prep Kit – sets A and B (Illumina, Cat. No. RS-122-2101 and RS-122-2102). Resulting short fragment libraries were checked for quality and quantity using the Bioanalyzer (Agilent) and Qubit Fluorometer (ThermoFisher). Libraries were sequenced as 50bp single reads on a high-output flow cell using the Illumina HiSeq 2500

instrument. Following sequencing, Illumina Primary Analysis version RTA 1.18.64 and

bcl2fastq2 v2.18.0.12 were run to demultiplex reads for all libraries and generate FASTQ files.

LAN5, NB1643 & SHSY5Y in transplanted & migrated bulk RNA-seq

Cells were sorted directly into kit lysis buffer and processed following manufacturer's directions

for cDNA synthesis using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara,

634891). Subsequent library preparation was performed according to manufacturer's directions

for the Nextera XT kit (Illumina, FC-131-1096). Resulting short fragment libraries were checked

for quality and quantity using the Bioanalyzer (Agilent) and Qubit Fluorometer (ThermoFisher).

Libraries were and sequenced as 50bp single reads on a high-output flow cell using the Illumina

HiSeq 2500 instrument. Following sequencing, Illumina Primary Analysis version RTA 1.18.64

and bcl2fastq2 v2.18.0.12 were run to demultiplex reads for all libraries and generate FASTQ

files.

SHSY5Y in culture scRNA-seq

Dissociated cells resuspended in chilled 1X DEPC PBS were assessed for concentration and

viability using a Cellometer Auto T4 cell counter (Nexcelom). Cells were loaded on a Chromium

Single Cell Controller (10x Genomics, Pleasanton, CA) based on live cell concentration.

Libraries were prepared using the Chromium Single Cell 3' Library & Gel Bead Kit v2 (10x

Genomics, PN-120237) according to manufacturer's directions. Resulting short fragment

libraries were checked for quality and quantity using a Bioanalyzer (Agilent) and Qubit

Fluorometer (ThermoFisher). Libraries were pooled and sequenced on rapid mode flow cells

using the Illumina HiSeq 2500 instrument with the following paired read lengths: 26bp Read 1, 8bp i7 index, and 98bp Read 2. Primary analysis was performed with 10x Genomics' Cell Ranger software. Clustering and differential expression between clusters was determined by 10x Genomics' Loupe cell browser (v3.1.0).

LAN5 in culture and hanging drop scRNA-seq

Dissociated cells resuspended in chilled 1X DEPC PBS were assessed for concentration and viability using a Cellometer Auto T4 cell counter (Nexcelom). Cells, having been deemed to be 63-87% viable, were loaded on a Chromium Single Cell Controller (10x Genomics, Pleasanton, CA) based on live cell concentration. Libraries were prepared using the Chromium Single Cell 3' Library & Gel Bead Kit v2 (10x Genomics, PN-120237) according to manufacturer's directions. Resulting short fragment libraries were checked for quality and quantity using a Bioanalyzer (Agilent) and Qubit Fluorometer (ThermoFisher). Libraries were pooled and sequenced on 1 rapid mode flow cell using the Illumina HiSeq 2500 instrument with the following paired read lengths: 26bp Read 1, 8bp i7 index, and 98bp Read 2. Primary analysis was performed with 10x Genomics' Cell Ranger software. Clustering and differential expression between clusters was determined by 10x Genomics' Loupe cell browser (v3.1.0).

FIGURE LEGENDS

FIGURE 1: Human neuroblastoma (LAN5, SHSY5Y, NB1643) cell invasion patterns in vitro and in vivo in a chick embryo transplant model. (A) In vitro culture of LAN5 and (B) measurement of the displacement of LAN5 cells in culture over time. (C) TrkB/BDNF expression ranges for LAN5, SHSY5Y, and NB1643 human neuroblastoma cells in culture. (D) Chick embryo transplant model schematic, including hanging drop culture and transplant into chick embryo trunk neural tube (Steps 1,2), imaging of transplanted neuroblastoma cell invasion patterns after egg re-incubation (Step 3), and schematic of scoring for invasive neuroblastoma cell positions in vivo and in transverse tissue sections (Step 4a,b). (E,I,M) LAN5,SHSY5HY, and NB1643 cell invasion patterns (orange-colored cells) in a typical chick embryo transplant 48hrs post-transplantation and egg re-incubation with HNK-1 staining of the host chick neural crest cells (blue), respectively. (F,J,N) Neuroblastoma cell count versus position after invasion into the chick embryonic trunk neural crest microenvironment. (G,K,O) Percent of neuroblastoma cells migrated onto either the rostral (similar to host neural crest cells) or caudal somite pathways. (H,L,P) Percent of neuroblastoma cells identified in the dorsal and ventral tissue structures of the chick embryonic trunk neural crest microenvironment measured at 48, 72, and 96 hrs after transplantation,egg re-incubation and cryosectioning. NT=neural tube, NTL=neural tube lumen, DL=dorsolateral migratory pathway, UDL=upper dorsoventral migratory pathway between the dorsal neural tube and dorsal root ganglia, DRG=dorsal root ganglia, LDL=lower dorsoventral migratory pathway, VR=ventral root, SG=sympathetic ganglia, DA=dorsal aorta, N=notochord. The scalebars are 100um in (E,I,M).

FIGURE 2: Manipulation of TrkB in human neuroblastoma cells reveals significant changes in the cell invasion patterns after transplantation into the chick embryonic trunk neural crest microenvironment. (A) Invasion pattern of LAN5 trkB shRNA in a typical chick

embryo 48 hrs after transplantation with a subset of the small number of escaping fluorescently labeled cells circled and (B) higher magnification of the transplanted cells in a different embryo. (C) Measurement of the number of invading cells comparing LAN5 WT and trkB shRNA embryos. (D) Invasion pattern of SHSY5Y trkB shRNA in a typical chick embryo 48 hrs after transplantation with a subset of escaping cells circled and (E) higher magnification of the transplanted cells in a different embryo. (F) Measurement of the number of invading cells comparing SHSY5Y WT and trkB shRNA embryos. (G) Invasion pattern of NB-1643 FL trkB in a typical chick embryo 48 hrs after transplantation with a subset of the small number of escaping cells circled and (H) higher magnification of the transplanted cells in a different embryo. (I) Measurement of the number of invading cells comparing NB-1643 WT and FL trkB embryos. Scalebars are 100um in A,D,G and 50um in B,E,H. nt=neural tube, s=somite. HNK1 labels host migrating neural crest cells (Red) and DAPI (all cell nuclei in host embryos) in A,D,G.

FIGURE 3: Correlation analysis, comparison of gene expression changes and GO Term Enrichment of the three human neuroblastoma cell lines (LAN5, SHSY5Y, NB-1643) in culture and after exposure to the chick embryonic trunk neural crest microenvironment. (A) Correlation analysis of the three cell lines (SHSY5Y, LAN5, NB-1643) from culture and after embryonic exposure, (B) comparison of genes changed after exposure to the embryonic microenvironment from each cell line, and (C) Go Term Enrichment analysis.

FIGURE 4: Analysis of tumor initiating and stem cell gene expression changes in human neuroblastoma cells after exposure to the chick embryonic trunk neural crest microenvironment. (A) Changes in expression of typical tumor initiation markers in LAN5, (B) SHSY5Y, and (C) NB-1643 cells. (D) Changes in expression of typical stem cell markers in

LAN5, (E) SHSY5Y, and (F) NB-1643 cells. (G) Single cell RNAseq cluster analysis of LAN5, SHSY5Y and NB-1643 cells. (Needs finishing).

FIGURE 5: RNAseq reveals gene expression differences in non-migratory and invasive cells after exposure to the embryonic trunk neural crest microenvironment. (A) Correlation matrix analysis. (B) Comparison of the set of genes in common across all 3 cells lines show 328 genes in common in non-migratory cells and a common set of 1,677 genes in invasive cell populations that were altered after embryonic exposure in all 3 cell lines. (C) GO Term enrichment analysis showed that the set of 328 genes categorized into cell adhesion and development. (D) GO Term analysis of the invasive cell populations showed enrichment for neurogenesis, differentiation, guidance and morphogenesis involved in migration. (E) Go Term alignment and percent of annotations that were changed in either the non-migrated or invasive populations compared to random signatures genes.

FIGURE 6: Computational model predictions of human neuroblastoma cells that remained at the transplant site show improved disease outcome. (A) Model input genes (MDK, Alk, BDNF, trkA, trkB, NGF) extracted from LAN5, SHSY5Y, NB-1643 cells in culture (WT), transplant (remained at the transplant site), and migrated (invasive into the chick embryonic trunk neural crest microenvironment). (B) Model simulation output in four cell states (Differentiation, Apoptosis, Angiogenesis, and Proliferation) with the steady state (Ss) outcomes from the model simulations and (C) Predicted outcome from the model simulations.

SUPPLEMENTAL FIGURE S1: (A) Hanging drop culture of LAN5 (green) and Caspase-3 (red) staining with arrowhead pointing at Caspase-3 positive cell (orange) and (B) average number of Caspase-3 positive cells per hanging drop cluster. (C) CHLA-10 (green) transplant into chick

embryo trunk neural tube with rostral (r) and caudal (c) portions of the somite labeled. The

scalebar is 50um.

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In contrast, TRKB expression is associated with a poor prognosis in NB, present in >50% of high-risk cases and correlates with *MYCN* amplification.[45] TRKB activation leads to enhanced oncogenic potential in NB cells. When BDNF, the TRKB ligand, is applied to *MYCN*-amplified NB cells, there is improved cell survival and neurite growth.[45] TRKB is also associated with the angiogenic factors, VEGF and bFGF, suggesting that it may promote angiogenesis and metastatic ability.[25,98,99] Furthermore, TRKB expressing cell lines are less sensitive to doxorubicin, etoposide, and cisplatin, suggesting that TRKB may abrogate response to chemotherapy.[100] The association between TRKB/BDNF and cell survival, angiogenesis, metastasis, and drug resistance suggests that TRKB in NB may be a useful therapeutic target.[76,101]